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Editor
FRANK J. ORLAND

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EDITORIAL VIEWS AND NEWS

As the JOURNAL goes to press it is most gratifying to mention that the first part of the William J. Gies Foundation Grant has just been received by the I.A.D.R. Treasury to cover the cost of additional pages in the JOURNAL OF DENTAL RESEARCH. At the recent meeting of the I.A.D.R. in Detroit, the Gies Foundation award was announced jointly by Reidar F. Sognnaes, then I.A.D.R. President, and Bissell B. Palmer, President of the William J. Gies Foundation for the Advancement of Dentistry, Inc., New York. The Gies Foundation was established in honor of William J. Gies, Professor of Biochemistry at Columbia University, founder of the JOURNAL OF DENTAL RESEARCH in 1919, and co-founder of the International Association for Dental Research in 1920. It is certainly commendable and fitting that this Fund is now able to assist this JOURNAL, nurtured in its early years by William Gies himself.

With the additional pages appearing in this issue as well as in the last issue, the lag period of papers has been materially reduced. Thus, it will be noted, that with more pages and with rigorous editing, this June issue is comprised of papers received for publication in late 1957 or in early 1958. A 6-month processing period is comparable to that of most other conventional scientific journals.

New Section in the Journal.—To expedite research publication even further, it is planned to initiate a new section in this JOURNAL. This new section would contain brief research notes under the general title of "Annotations." It would have its own chronology, publishing items in the order of receipt. Such concise notes, of necessity, would be limited to 1 page or about 400 words. There is ample precedent for such short papers in similar sections in other scientific journals. Nevertheless, such annotations should be meritorious in content and written in an acceptable manner. They may also be reviewed by the Advisory Editorial Board just as regular manuscripts are being evaluated. Rapidity of publication and therefore availability to readers of the JOURNAL OF DENTAL RESEARCH would be their greatest attribute. Thus, the JOURNAL is now open (on a trial basis) for publication of concise research notes. All this is in keeping with the views expressed by the President of the International Association of Dental Research, Ned B. Williams. His full inaugural address will appear with the transactions of the Detroit meeting in a subsequent issue of the JOURNAL. The previous inaugural address of Reidar F. Sognnaes, given at the 35th General Meeting of the I.A.D.R. in Atlantic City, appears in this issue.

It should be mentioned that H. B. G. Robinson edited about half of the papers appearing in this present issue and due credit should be given to him. Thus it is with this June number that the JOURNAL OF DENTAL RESEARCH is completing the transition to the new staff. The new Associate Editors, David Mitchell and John Macdonald, are currently in charge of various aspects of JOURNAL circulation among other things. Certain activities of the business manager's office are now being handled by the I.A.D.R. Secretary-Treasurer, Dan Burrill. The Assistant to the Editor, Vernon Brightman, as well as the Manuscript Secretary, Dorothy Herbison, are both members of the Zoller Memorial Dental Clinic at the University of Chicago, as is also the Editor. The Advisory Editorial Board, consisting of 19 members at present, is functioning effectively in the help it provides reviewing manuscripts. It remains for the Editor to co-ordinate the several phases of journal activity, as well as to integrate and work out any and all aspects of the actual production of the JOURNAL.

In order for the new Editor to obtain directly the views of at least a portion of the reading audience of the JOURNAL, a questionnaire is to be sent to all I.A.D.R. members in the near future. The results may help in formulating future editorial policy.

FRANK J. ORLAND
EDITOR

PRESIDENTIAL INAUGURAL ADDRESS

A PRECIOUS HERITAGE

Footnotes on Ivory Art and Dental Science

REIDAR F. SOGNNAES

Harvard School of Dental Medicine, Boston, Mass.

IF ANYONE should happen to have heard of my going abroad last summer, a family of six, and a camera slung over the shoulder, I regret to say that my photographs were not good enough to save for this day.

If—as a next best choice—you should be expecting a dissertation on the dental enamel, the surface of which I have been scratching for a few years, I hasten to say that my remarks on this occasion will go a little deeper—a fraction of an inch deeper at any rate—into the underlying dentin or ivory core of teeth and tusks.

What I have to say will, nevertheless, touch upon faraway lands and bygone days. For the story of ivory begins in antiquity, when man first discovered the dexterity of the hands, the beauty of the teeth, and the satisfaction derived from the dental art.

Ivory art is as unique in substance as it is antique in origin. In the history of art, this magnificent biological product has been derived from the hairy mammoth, and from the elephant, whose formidable tusks, the central incisors, are spread apart like canines by the protruding trunk. Ivory objects of art and utility have also been carved from the dentin of the mandibular teeth of the sperm whale, from the canines of the walrus and hippopotamus, from the fabulous "unicorn" tooth of the narwhal, and, to a lesser extent, from the teeth of other animals, ranging from the polar bear of the arctic to the boar of the tropics.

The origin of ivory art can be traced further back into human history than any other form of sculpture. It can be followed from prehistoric ages to predynastic Egyptian, prehistoric Assyrian, ancient Oriental, Hebrew, Greek, Roman and Christian cultures, to recent times.

The first drawings of the mammoth on the walls of the prehistoric home of the cave dwellers in the Dordogne of France indicate that the tusks and proboscis, with its fingerlike projections, were the center of cave man's attention. The cave dweller's first portrait was carved in ivory, the first manual aptitude test. Man's first engravings were made upon the slab of a tusk and depicted the contour of his contemporary, the hairy mammoth, the maker of ivory.

Delivered before the 35th Annual Meeting of the International Association for Dental Research in Atlantic City, N. J., on March 22, 1957.

As we cross the sea of man's cultural evolution, we still sense the ripples set in motion in antiquity. The tools and the themes have changed. But a similar motivation probably led to the most recent ivory art: the scrimshaw carvings of the American whalers of last century. Their object was the sperm whale ivory, the subject their ships and sweethearts on faraway shores, from New Bedford, Massachusetts, to the Pacific Islands.

Between these two extremes—the carvings of the cave man and those of the whaler—nearly every conceivable object of art and utility has at one time or another been fashioned from the substance of the teeth.

For ivory—in times past—there was no place like home. For the lady of the house, there were ivory jewelry boxes, bracelets, rings, combs, and hairpins. If she was the wife of a whaler, even her corset was fortified with stays of ivory and jawbone of the whale. For the scholar of the house, ivory decorated the armrests of his writing desk and served as his penholder and ink receptacle. For contemplation he had ivory pins with which to scratch his head.

Excavations of prehistoric graves have yielded ivory dice which have not been thrown for more than 10,000 years. King Tutankhamen was entombed with ivory dice for the life hereafter. Poker did not wait for plastics. Ivory playing cards were once made of thin painted slabs of ivory. Chess, the game of games, inspired our ancestors to ivory carvings now treasured as objects of art rather than play. Billiards depended on the resiliency of ivory. It was once tried on the head of golf clubs. In the musical department, there were ivory flutes and trumpets in size and shape matching whole tusks. Ivory has decorated numerous stringed instruments. The ivory plectrum and castanet kept the rhythm of the ancient's "Rock and Roll."

But most remarkable of all dental traditions were the closely allied talismanic and curative powers attributed to teeth and ivory. Ivory combs were once thought to cure headache and restore hair. Powdered ivory was thought to do this, and more. It has been burned on the altars as incense. It has been fed to cattle to improve milk production. It has been mixed with honey for treatment of skin blemishes. It has been mixed with goat blood for relief of pain. Walrus ivory once took a high price as an antidote to poison and by the ancient Orientals was considered the material of choice for chopsticks. The Laplanders of northern Norway placed a heated tooth on their gum boils—a tooth for a toothache. The pill boxes and medicine chests of the ancients were made of ivory. In diagnosis, the shy Chinese ladies are said to have used nude ivory figures to point out the site of discomfort, hiding their own body behind a curtain.

And so we see that teeth and ivory through the ages have helped man's faith, and been conducive to his pleasure and well being. Before the advent of antibiotics and psychiatry, the substance of the teeth appears to have served as a sort of psychosomatic medicine; dental medicine, literally speaking.

It is not surprising that, in times past, the solid yet mellow, soft, silky appearance of polished ivory became the poetic synonym of bodily strength and beauty. "Thy neck is as a tower of ivory," sings the Song of Solomon (vii: 4). In a lighter vein, Homer exclaims in the Odyssey (XVIII): "From limb to

limb an air majestic sheds, and pure ivory over her buzom spreads." And yet the classical writers were fully aware of the fact that the quality of ivory—like that of the skin you love to touch—is subject to considerable variations.

This brings me to my first footnote of dental science—*variety in quality.* Our first literature reference is to the Athenian second century philosopher, Philostratus, who, in the *Life of Apollonius* (Vol. I, Book II, Chapter XIII), described three qualitative types of ivory: *First:* "The tusks of the marsh elephant," he wrote, "are dark in color and porous and difficult to work because they are hollowed out into many cavities, and often knots are formed in them which oppose difficulties to the craftman's tool." These marsh elephants, he added, are "considered to be stupid and idle. . . ." *Second:* ". . . the tusks of the mountain kind, though smaller than these, are very white, and there is nothing about them difficult to work." Nevertheless, he stated that these mountain elephants are regarded as "wicked and treacherous and, unless they want something, not to be relied upon by man." *Third:* ". . . best of all are the tusks of the elephants of the plain, for these are very large and very white and so pleasant to turn and carve that the hand can shape them into whatever it likes; . . ." In this case, Philostratus went out of his way to emphasize the coincidence between dental and mental superiority, when he submitted: "The elephants of the plain are said to be good and tractable, and fond of learning tricks; for they will write (*sic*) and dance, and will sway themselves to and fro and leap up and down from the ground to the sound of the flute." I assume it must have been an ivory flute.

Such variations in the quality of the tusks became of paramount importance to the ivory trader and in turn to the ivory turner. The ancient Chinese carver was fully aware of such variations and considered the African ivory superior to the Asiatic variety.

The life habits of these two species differ to some extent. The Indian elephant is said to have a certain aversion to exposing itself to the sun. It stays away from the plains, preferring the shady forests, whereas the African elephant not only roams the plains and the lowland, but also has been found to seek the open hills and the stronger sun of extremely high altitudes.

A complicated mixture of influences is at work because of the wide area of the African continent and the wide range of its climatic conditions, temperature, latitude, altitude, rainfall, sun exposure, and vegetation. For these reasons, one is not surprised to find exceptions to the rule of thumb, according to which ivory from the highlands of West Africa is better than that from the low-leveled East and North.

High on the list of quality stands the ivory from the Cameroon district, the high mountain region of equatorial West Africa which exhibits a vegetation as varied as can be found any place between the equator and the Arctic Circle. The elevated portions of the Cameroon not only represent a rich food supply but are also continuously as near the sun as any feeding land on earth.

I need hardly remind this audience of the United States statistics on the quality of human teeth which has been correlated with latitude and sun exposure. Going north from the Mexican Gulf to the Canadian border, you will

recall that Mills[1] demonstrated an increase in tooth decay by 15 DMF teeth per 100 children for each latitude. Subsequently, it was shown by East[2] that the DMF rate rose by 40 per cent as he moved from United States localities with 3,000 hours of annual sunshine to those with 2,000.

In more recent studies we have become impressed by the fact that human teeth from various parts of the Old and New World, representing a span of time from prehistoric cave man to modern man, almost universally exhibit microscopic evidence of faulty calcifications.[3] In this respect, human teeth appear very inferior to those of the lower primate, e.g., those of the rhesus monkey basking in the sun of India's treetops. The terrestrial apes seem to occupy an intermediary position[4] with teeth less perfect than those of the monkey, but generally superior to those of *Homo sapiens.*

Returning to the elephant tusk, our recent microradiographic examination of thin ground sections of ivory show that this substance is divided into broad zones of very high and very low degrees of calcification. This explains the fact that the average specific gravity of ivory is only about 1.80 to 1.90. Furthermore, it seems possible that the variations in ivory quality noted by Pliny and Philostratus may now be accounted for, in part, by different proportions in the width of these two zones and, in part, by the presence of completely uncalcified interglobular dentin substance similar to that observed in poorly calcified anthropoid teeth.

So much for the variety in quality of ivory; now for *variety in form.*

Long before the advent of airbrasive, ultrasonic, and high-speed tools for preparation of teeth, the ancient artist accomplished the most intricate ivory carvings. Most remarkable were the so-called "magic balls," the size of billiard balls, yet containing artistically carved spheres, one within the other, up to a dozen or more, the record being 28. For such fine work, the ancient Orientals developed hand instruments, many of which resemble those from a modern dental supply catalogue.

But unlike many other raw materials for art and utility objects—ranging from pottery to plastic—ivory can neither be molded, melted, cast, nor hammered into new form by any known tools of modern man. How then are we to account for the great variety of ivory objects left to us by the Ancients, especially the wide range in over-all dimension and shape?

The first century Roman naturalist, Pliny, in his *Natural History,* described among the tiniest objects on earth some extremely minute animals, not microbes to be sure, but animals carved in ivory, so minute in size that they had to be placed on a dark hair to be seen. Contrast this to the classical description of the chryselephantine statues of Phidias, representing Jupiter and Minerva at Olympia and Athena of the Parthenon. These stood over 40 feet high. We know that it was ivory which rendered the beauty to these. It appears that the carving of each finger of these gigantic statues must have required a whole elephant tusk.

Between these two extremes of Pliny and Phidias, one could enumerate ivory objects of nearly every conceivable size and shape.

The houses of ivory have perished, as the Bible predicted (Amos, iii: 15), but we still possess several examples of ivory thrones. An ivory-decorated ecclesiastical chair and footstool were found entombed with King Tutankhamen of ancient Egypt. But this was merely a rehearsal for greater things to come. The ivory throne of King Solomon was not built in a day, let alone the ivory house of Ahab, successor to the ivory throne.

From the Christian era originated the sixth century ivory throne of Archbishop Maximanus, which stands in the Cathedral of Ravenna (A.D. 546). The ancients knew how to glue ivory and gold with a concoction made of the fish's swim bladder. From more recent times, A.D. 1671, we can still behold the exquisite beauty of the Coronation Chair of Denmark's King Christian V, carved out of narwhal tusks and standing in the Riddersal of the Rosenberg Castle in Copenhagen.

The houses of worship have been adorned with ivory altars, candle holders, sceptre or crosier, the pectoral cross, and the liturgical comb and fan, holy water vats, ivory patera, pax and pyxides for the consecrated elements of the Lord's Supper, and ivory caskets containing the bony relics of the sacred saints. Ivory tablets and double-tablets, the diptychs, have recorded nearly every event in the Holy Scriptures, and once served as a church register of births and deaths. According to the classical writers, the funeral couch, pyre, and bier were decorated with ivory, worth its weight in gold, from cradle to grave.

Being fit for a king, it is not surprising that ivory has served as the gift of choice "for those who have everything." King Darius received annual ivory gifts from the Ethiopians, says Herodotus. The merchants of De-dan "brought thee for a present horns of ivory and ebony," says the Bible (Ezekiel, xxvii: 15). The ivory of walrus teeth was used as a means for obtaining royal favors, says the Norse Saga. King Tutankhamen for the life hereafter was entombed with ivory implements ranging from ivory gaming boards to an ivory-decorated bed; a heavenly resting place, indeed.

The prehistoric Egyptians carved human figures from the whole elephant tusks, the tapering incisal end giving a characteristic pinhead appearance to their earliest sculpture. During the Christian culture, the holy figures of the Madonna and Child, as well as of the Lord, were similarly carved from the broader portion of whole tusks, necessitating a curved position of the statues. It is interesting to note that this attractively humble position was to be maintained in more recent sculpture of wood, even though no longer demanded by the shape of the raw materials.

More mysterious are the large, flat ivory plates, book covers, diptychs, triptychs, etc., some of which in dimension exceeded the natural diameter of the solid portion of the elephant tusks. Early references to ivory art contain strong suggestions that the ancient artists possessed some tricks of the trade whereby

they succeeded not only in cutting thin ivory slabs, but also in softening, bending, and rehardening ivory slabs into desired shape beyond the anatomy provided by nature.

For the earliest writings on this subject from a technical point of view, we have to turn to the well-informed artist-monk, Theophilus, of the eleventh century.[5] "Should you wish to bend ivory and ornament it, take sulphate of potash (*glumen rotundum*), fossil salt (*sal gemma*), and vitriol (*calcanthum*); these are ground with very sharp vinegar in a brass mortar. Into this mixture the ivory is placed for three days and nights. This being done, you will hollow out a piece of wood, as you please. The ivory being then placed in the hollow you direct it, and will bend it to your will."

Such a buffered solution of acetic acid was probably a more effective decalcifier than the softening solution used by the Eskimos of Alaska, who, while carving, would soak the dense walrus ivory in their own urine intermittently during elaboration of the artistic workmanship.[6]

For the rehardening of such softened and bent ivory, there is only one conceivably effective concoction which the artist appears to have left to posterity, namely that from the twelfth century English manuscript, *Mappae Clavicula* by Sir Thomas Phillips[7]:

Quicklime	2 parts
Pounded tile	1 part
Torn tow (*stuppa scissae*)	1 part
Oil	1 part
Mix with lye made of elm bark	

In this alkaline solution of calcium oxide, silicate, and polysaccharide, the ivory was to be placed for 3 days and nights. But this was art, not science. No one knows the unpublished secret formulas which the ancient artists, after trial, error and possibly success, may have kept to themselves for rehardening ivory. Yet, the German ivory art historian, Pelka,[8] concludes that (author's translation), "The definite manner in which these references have appeared makes it probable that the ancients, in fact, did possess such a technique, the knowledge of which has become lost."

A clue to this mystery may be found in our recent microradiographic studies of ivory, which suggest that this substance of the elephant tusks contains wide bands alternating between a very high and a very low degree of calcification. Possibly the ancients may have achieved a partial or preferential demineralization of these semisoft areas before bending the material. As yet, there is no adequate knowledge to explain the mechanism of rehardening of dentin. Yet, there is every reason to expect important information in years to come, regarding reconstitution and remineralization of a partially softened organic matrix. It has been demonstrated experimentally that collagen broken up into its submicroscopic units by acid, can be reconstituted by certain salts and polysaccharides. Also, it has been suggested that in calcification there is a precise relationship between the ultra-structural periodicity of collagen and the deposition of the inorganic crystal units. Basic information of this nature will

undoubtedly some day remove this subject from the realm of art and philosophy, make it a matter of respectable science, and permit intelligent application to our everyday problems.

My third and last footnote of dental science concerns the mystery of certain *voids in ivory art.*

In a previous paragraph it was stated that ivory art is an important link in the chain of cultural evolution. Nevertheless, within certain geographical regions and cultural periods, there are certain voids in the history of this art. This could simply be explained by accepting the discontinuity of the art, were it not for the written word and other documentary evidence, to the contrary. No adequate explanation has been advanced to fully account for these barren periods.

As we all know, the teeth are the best preserved remains of the body after death. Yet, various post-mortem dental changes can oftentimes be demonstrated in teeth which have been buried in the ground. The prevalence and nature of such post-mortem changes became evident in the course of a study of exhumed human teeth originating from paleolithic Palestine, prehistoric Egypt, Greece, ancient Iceland, Norway, and Central America.[9] The destruction could be seen both in x-rays and microscopic sections.

Minute earthly organisms, presumably fungi, have been shown to have great appetite for the collagenous matrix of dentin after breaking down the inorganic lime salts, the apatite crystals. But the latter are only the appetizers, as it were, because the enamel is of no interest to them as a source of food, its high apatite content notwithstanding. Sometimes the shells of enamel are the only remnants of exhumed ancient teeth.

By the same token, I believe that similar types of destruction must have befallen objects of art made from the dentin of animals—including the ivory from mammoths and elephants, the most common variety of dentin used in carvings.

No one can guess at the earthly sojourns which ancient ivory art objects may have fallen prey to in times past, but I submit that this readily demonstrable post-mortem deterioration constitutes circumstantial evidence to account for the mystery of some of the voids in ivory art. The tusks, unfortunately, have no telltale enamel to leave behind. But between perfect preservation and complete destruction, there are ivory objects of intermediary preservation. One of the earliest is a carving by cave man of cave woman, who looks literally moth-eaten, the so-called Venus of Brassempouy, who has lost her head, if she ever had one. One gains the anatomical impression that the head was not cave man's main interest. Boys were and will be boys!

Despite these voids in a vanishing art, we are able to trace man's interest in tusks and teeth from the time of cave man to the advent of modern dentistry.

Yes, we would not wish to forget that it was ivory which in times past served as the material of choice for reconstruction of lost teeth. This material, whether from elephant or hippopotamus, was the last word in dental materials

at the time of the first President of the United States. It was a great professional pride in a present, which now is past, that prompted the New England minister-dentist-poet, Solyman Brown, a century ago, to phrase the biological origin of this dental material in poetry:

> In climes remote, where sacred Ganges flows
> From Tibet's mountains of eternal snows,
> Or far beyond the golden Gambia's source,
> Where Lander sought the Niger's mystic course;
> The lordly elephant, in hoary pride,
> Toils through successive ages to provide
> The ivory tusk; the fertilizing Nile
> Breeds the huge Hippopotamus, whose spoil
> Supplies new treasures;—and the ocean wave
> Nurtures the sea calf in his rocky cave,
> To furnish fit materials to impart
> Increased importance to the favorite art.

The fact that anyone among us today can do a great deal better than the foremost dentist could do with ivory for Washington's "bridge" is a testimony to the great advances made in dental materials, their processing methods, and application to the healing art. I have no doubt that the margin between success and failure which still is so narrow in many areas of dentistry will be continuously changed by the progressive research of our dental materials group.

The sciences which guide the biological and technical progress of dentistry must go hand in hand. It is a matter of attitude as well as aptitude. The pendulum of educational philosophy must not be allowed to settle at either extreme. For there is a positive asset inherent in the fact that dentistry combines the pleasures and problems of art and science. ***This is our precious heritage.***

On the cover of the program for this annual meeting are cited the three last wishes expressed to us by the late Dr. William J. Gies, father of our Association and founder of our JOURNAL. He left this guiding postscript to a rational, industrious, and useful life: "May the Association grow continuously in scientific vigor, in professional influence, and in public accreditation." (From letter of May 8, 1956).

It is gratifying to observe, as we measure the growth to date, that Dr. Gies was to see so much of his dream already come true:

1. Growth in *scientific vigor,* his first wish, is amply demonstrated by the tremendous increase in scientific reports presented at these annual conventions: climbing from 2 reports at the first annual meeting in 1922, to 103 in 1932, reaching 200 in 1954, and a record-breaking 306 at this 1957 convention, so many that 3 to 4 sections have had to be run simultaneously, covering 18 different categories, including the growing dental material group, and requiring nearly 50 reports to be read by title (Fig. 1).

2. Growth in *professional influence,* his second wish, is equally apparent from the increase in membership, which has climbed from the 40 founding members early in 1920 to 74 in 1928, 421 in 1938, 610 in 1948, and as we convened this 1957 meeting a total 861, including 714 in the North American Division, and 147 in the growing membership abroad (Fig. 2).

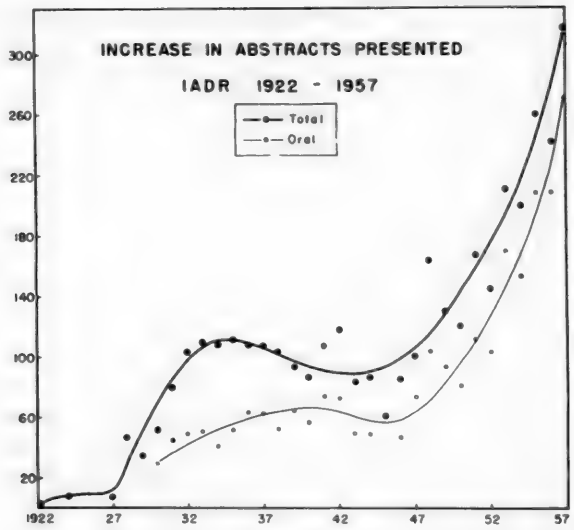

Fig. 1.

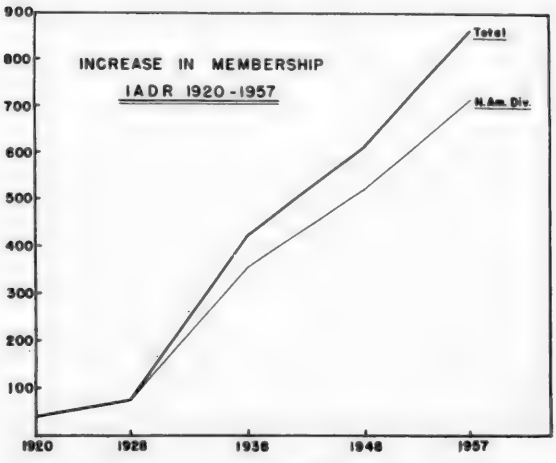

Fig. 2.

3. Growth in *public accreditation* was Dr. Gies' third and last wish. An important function in bringing the message of dental science beyond the circle of these annual reunions, is served by our JOURNAL OF DENTAL RESEARCH. While the quantity of published research papers is not alone an adequate

yardstick of science, it is a great credit to Dr. William Gies and Dr. Hamilton Robinson, its two devoted editors who carried this difficult load for about two decades each, that the number of articles published per year in the JOURNAL quadrupled from 31 in 1919 to 129 in the last complete volume of 1956. Much remains to be done in bringing before scientists in other fields, and before the public at large, the message of dental research, and to interpret its meaning and importance to health and welfare. Dr. Gies himself was a living testimony to the inspired curiosity which dental research can instill into scientists in other fields, once they are acquainted with the problems, fascination, and challenge of dental science, education, and practice. If encouraged, this development is bound to grow. If welcomed, there is nothing which succeeds like success.

Mindful of the high goals expressed in the last wishes of our founder, I am deeply cognizant of the honor and obligations inherent in serving this Association, grateful for the privilege to do so, and confident that the Association will grow continuously on the momentum of a vigorous past and in the tradition of a precious heritage.

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IN VITRO STUDIES OF TOOTH GERM AND PULP DEVELOPMENT

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THE in vitro cultivation of fetal tooth elements under the usual culturing environments, as with most tissues, usually results in rapid unorganized growth with a preponderance of fibroblastic activity. This rapid growth ordinarily takes place at the expense of tissue or organ differentiation; the two types of development are apparently incompatible and ordinarily not able to occur simultaneously.

Fortunately, it is often possible, by comparatively simple adjustments in environmental conditions, to encourage the migration or growth of certain specific cell types or tissues over others, and to induce at least a partial differentiation of some of the fetal elements. Such technic has been used by us in previous dental tissue studies,[1, 2] and by others[3, 4] and is currently being continued in our investigations of the development and maturation of tooth germs, fetal enamel organs, and pulp.

MATERIALS AND METHODS

Incisor and molar tooth germs were removed from 16- to 20-day albino rat fetuses and unweaned rats under a dissecting microscope. Some of the tooth germs were divided into enamel organs and papillae which were cultured separately. Papillae or pulps, chiefly from the incisors, were grown whole or as labial, lingual, and intermediate strips. Some of these strips were further subdivided into anterior and posterior (apical) halves. Whole tooth germs were cultured in 3.5 cm. Carrel flasks adapted to a roller mechanism. Enamel organs and pulps were also cultured in Carrel flasks but generally without rotation, since comparatively short periods were sufficient for the growth of small fragments and the observation of specific cell types.

In general, standard culturing methods were followed.[5] All tissues were explanted into coagulated media of either Hanks' or Earle's balanced salt solutions, chicken plasma, and chick embryo extract, or rat embryo extract. Chick extract proved as satisfactory as rat extract when used in a fresh state or, if frozen, shortly after the first thawing. A liquid nutrient essentially similar to the coagulated medium, but with unfiltered horse serum in place of plasma, was

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added and changed at 2- to 3-day intervals. Cultures were ordinarily maintained in this media and nutrient, at pH 7.0 to pH 7.2, and at temperatures of 36° to 37° C.

For experimental purposes, nutrient was varied by reducing or increasing the serum or embryo extract fractions and by adding calcium, magnesium, and potassium in multiples of normal physiologic concentrations. Fragments of pituitary, thyroid, and parathyroid glands of females, from which fetuses were removed, were grown with the tooth germs of their fetuses. Temperatures were varied between 25° C. and 37° C.

RESULTS

Use of the combined Carrel flask-roller tube mechanism appeared to facilitate tooth germ development compared with nonrotated controls. Rotation generally prevented the flattening of tooth germs which usually occurred in long-term stationary cultures, and provided a more efficient, less damaging method than rocker flasks, etc., for nutrient and gas exchanges. In addition, less liquefaction of the coagulated medium occurred in the rotated flasks. Pituitary, thyroid, and parathyroid fragments, when combined and added in optimal amounts (about 1 part total hormone tissue to about 4 parts tooth germ tissue) also improved development. Increased development was also seen when calcium, magnesium, and potassium were added to normal media, provided total concentrations of these electrolytes were not more than twice physiologic levels. Greater concentration of these salts caused degeneration of the tooth germs, as did an increase in the ratio of glandular tissue. When all the above methods were combined with lower temperature environments (34°-35° C. rather than 36°-37° C.), the glass-grown rat molar tooth germs reached, in approximately 3 weeks of culturing, the most advanced development stages seen in these experiments. The preameloblast-ameloblast layer developed to the in vivo equivalent of 1 to 2 days of normal growth. The odontoblast layer of the papilla showed a similar degree of development. The predentin layer, or dentin matrix, at the dentinoenamel junction, widened to reach a developmental stage equal to that in an animal of 3 to 4 days postnatal age, or the equivalent of 7 to 8 days of normal growth (4 days prenatal and 4 days postnatal). In some instances, the earliest dentin matrix appeared to be laid down without or before the complete morphologic differentiation of the odontoblastic layer.

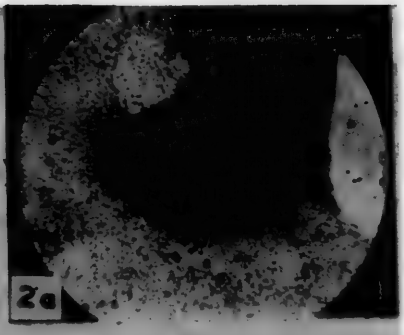
Continued exposure of earliest tooth germs, from 15- to 16-day fetuses, to still lower temperatures (30°-34° C.) often resulted in what appeared to be a loss of the characteristic cytology of the inner enamel epithelium. This region stained intensely with eosin, the cell borders were indistinct and the nuclei extremely elongated, pyknotic, and degenerating. It was difficult to determine whether this area was altered inner epithelium or part of the original papilla. If the latter, it was no longer demarcated from the enamel organ (Fig. 1).

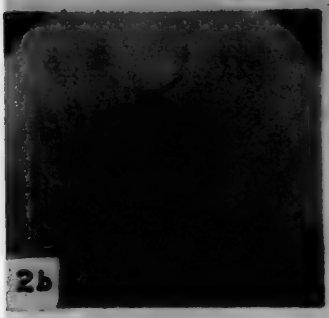
The region of the papilla frequently developed into structures closely resembling denticles (Figs. 2*a*, 2*b*). The acidophilic tissue at the inner enamel epithelium margin seemed to have been desquamated into the space previously occupied by the papilla, to form the concentric layers of the denticle (Fig. 3). The denticles consisted of concentric layers of homogeneously stained amorphous masses in which darker staining fibers were embedded. In incisors a single denticle often entirely replaced the early developing pulp, while in some molars multiple denticles replaced all the developing cusps (Fig. 4).

Late fetal pulps dissected from the surrounding enamel organs and grown in vitro frequently reacted to mild growth-inhibiting environments by cytologic differentiation. Temperatures of 30° to 34° C., low nutrient media, modified physiologic solutions, sometimes singly but more often when combined, tended to induce in the cultures early but recognizable narrow ribbonlike odontoblast or Tomes' fibers and Korff's fibers in 3 to 7 days. Under similar conditions, whole pulps dissected from the hard tissues of month-old rat incisors showed little organized outgrowth in vitro except for rectangular odontoblast-like cells from the anterior half or third of the labial surface (Fig. 5). However, when fetal or postnatal incisor pulps were cultured in growth accelerating environments (high nutrient content, frequent changes, 37.5° C., etc.) fibroblast-like cells migrated from both labial and lingual surfaces. In these instances outgrowth from the labial surface was consistently 2 to 3 times greater than that from the lingual surface (Figs. 6, 7).

Cultures of pulp from fetal rat incisors cut into longitudinal strips, i.e., labial, lingual, and middle segments, and grown under the mildly inhibiting conditions previously described, showed consistent growth patterns, most striking in the labial layer. When a labial strip was transected into anterior and posterior halves, a directional or polarized growth was seen. In cultures of the posterior labial half, with all epithelial margins removed, fibroblasts grew from both posterior and anterior ends, extending the strip of pulp chiefly in the posterior direction (Figs. 8, 9). Posterior growth was mainly responsible for approximately doubling the length of this posterior half in about 3 weeks. On the other hand, the anterior half of the labial strip contributed little to the over-all elongation. Lingual strips showed essentially similar growth patterns but took considerably longer for equivalent development.

Cultures of fetal enamel organs of rat incisors grown in temperatures of 20° to 25° C. for 15 to 18 hours, and then returned to normal body temperature environments, developed intense mitotic activity. Considerably more mitoses were stimulated by this method than is ordinarily seen in cultures kept at 36° C. The effects on the enamel organ were primarily on the growing apical end of the labial surface, from which a dense epithelial sheet proliferated posteriorly, i.e., apically, enclosing the apical end (Fig. 10). Parts of the sheet also turned anteriorly to invest the outer sides of the enamel organ. This posterior growth and investment was less complete and at a considerably slower rate when the culture was kept at 36° C. throughout the experiment.





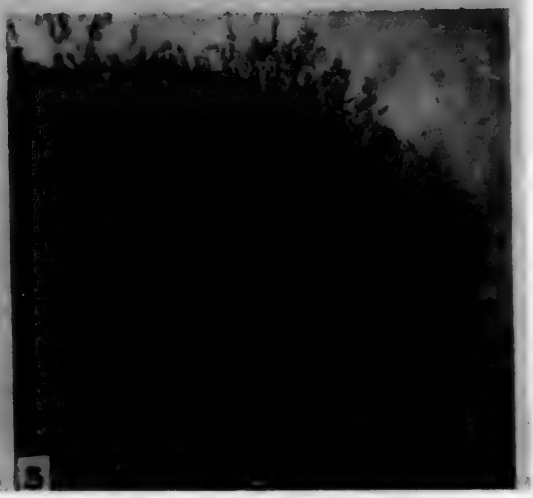


Fig. 1.—Altered inner enamel epithelium of a 15- to 16-day fetal rat tooth germ cultured for 8 days at 30° to 34° C. Elongated cells have replaced the more columnar type usually seen. (Fixed and stained.)

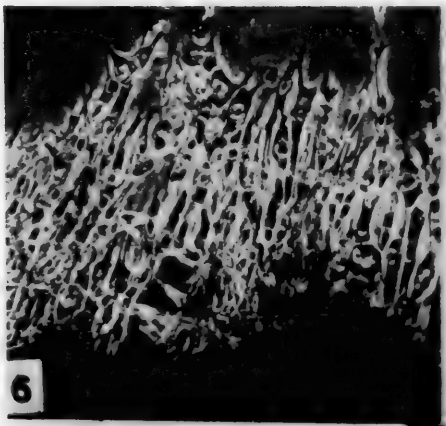
Fig. 2,*a*.—Denticle replacing papilla in an early fetal tooth germ. (Living culture, unstained.)

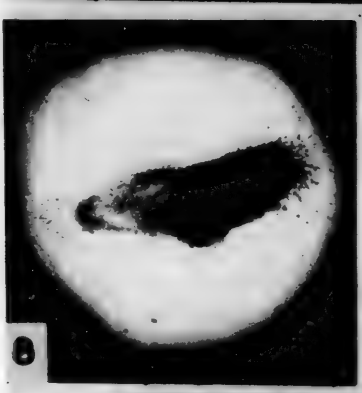
Fig. 2,*b*.—Papilla completely replaced by denticle in an early fetal tooth germ. (Fixed and stained.)

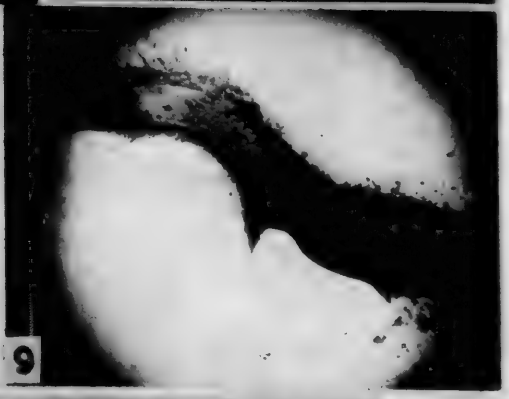
Fig. 3.—Typical desquamating layers forming a denticle similar to those in Figs. 1 and 2,*b*. (Fixed and stained.)

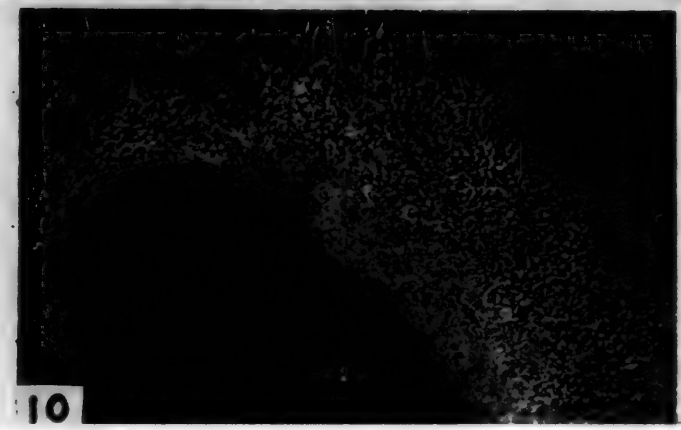
Fig. 4.—Multiple denticles replacing the developing pulps of a fetal rat molar tooth germ cultured at 30° to 34° C. (Living culture, unstained.)

Fig. 5.—Odontoblast-like cells growing only from the anterior labial margin of a month-old upper incisor pulp, removed from surrounding hard tissues and cultured for 7 days in a moderate growth-inhibiting environment. (Living culture, unstained.)









Figs. 6 and 7.—Unrestrained growth from a fetal rat incisor pulp cultured in a growth accelerating environment. Fig. 6—labial margin; Fig. 7—lingual margin. Labial surface outgrowth is 2 to 3 times greater than lingual growth. (Living cultures, unstained.)

Figs. 8. and 9.—Extent of growth of the posterior half of a labial strip of fetal rat incisor pulp, cultured under mildly inhibiting conditions. Growth is chiefly posteriorly (apically—left side of figure). Fig. 8 at 3 days of culturing; Fig. 9 at 8 days. (Living cultures, unstained.)

Fig. 10.—Apical lip of the labial surface of a fetal rat incisor enamel organ, showing proliferation of an epithelial sheet, growing inward (left side of figure) to meet the lingual side of the enamel organ and outward over the surface of the organ. (Living culture, unstained.)

DISCUSSION

The development of tooth germs in vitro, under current methods of cultivation, apparently can be brought only to a limited degree of maturation. The present results, perhaps a day further advanced than those of other investigators,[3, 6] emphasize this and point to the necessity for tissue culture environments more closely approximating conditions in vivo. The more advanced maturation of transplanted tooth germs in guinea pigs and rabbits[7] indicates that the organs can develop more completely away from their site of origin but require mechanisms present in living systems. These may well be enzymatic. Our use of hormonal tissues was a preliminary attempt to stimulate or reactivate some of these mechanisms. However, it seems probable that the specific enzymes or mechanism involved will have to be determined and added to the culture environment before tooth germs can be fully developed in vitro.

The fetal pulp of the continuously growing rat incisor appears to have an inherent capacity to determine its own conformation, at least initially, and perhaps to maintain this shape either independently or by supplementing a function of the enamel organ. The more rapid growth of the labial surface of the pulp compared with the lingual surface at this early stage, approximately concomitant with the onset of hard tissue formation, may be an important factor in forming the shape of the incisor. Also significant in this respect is the tendency of the continuously growing incisor pulp to elongate along its initial long axis while maintaining the convexity of its labial surface.

Many of the normal developmental processes of the maturing tooth germ can be observed in vitro and are less altered in this medium than might be supposed. One of the important differences between the 2 environments is the absence of confining bone and other structures in cultures. Yet the formation of a sheath similar to if not identical with Hertwig's sheath tends to occur much as it probably does in life. Even such cytologic elements as Tomes's and Korff's fibers have a reasonable resemblance to their stained appearance in histologic sections, provided unrestrained growth is prevented.

The replacement of the papilla area by structures resembling denticles in the tooth germs subjected to severe temperature variations was an unexpected development. Since both papilla and inner enamel epithelium were affected, it is difficult to classify the structures in these experiments on the basis of germ layer origin or site of development, although their resemblance to denticles is apparent. The homogeneously staining, amorphous central mass, interspersed with fibers, may be a predentin or precalcium matrix made up of continuously desquamating concentric layers from the peripheral tissue. The process appears to be an abortive attempt at premature calcification, coupled with or dependent on retrogressive changes induced by the severe temperature modification.

CONCLUSIONS

1. Variations of roller tube culturing methods prevented the flattening of tooth germs grown in vitro.

2. Endocrine gland fragments and electrolytes in optimum amounts improved the rate and extent of tooth germ maturation.

3. Lower temperature environments (35°-36° C.) seemed to produce the best differentiation of tooth germs and their components.

4. Severe temperature modifications (34° C. or less) induced denticle-like formations in early tooth germ papillae or pulp correlated with retrogressive changes in the inner enamel epithelium or periphery of the papilla.

5. Labial strips of the rat incisor pulp grew faster than lingual strips and mainly in a posterior (apical) direction.

6. The fetal pulp of the rat incisor seems to have an inherent capacity to determine its own conformation.

7. Development of tooth germs in vitro can be brought to only a limited degree of maturation with present-day culture methods.

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SODIUM AND POTASSIUM LEVELS OF HUMAN WHOLE STIMULATED SALIVA COLLECTED UNDER TWO FORMS OF STIMULATION FROM SUBJECTS IN A SELECT AGE GROUPING

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IT WAS the purpose of this investigation to study the effects of age and sex on salivary flow rate, sodium and potassium, and to determine the influence of flow rate on the levels of these cations. Analyses concerned with the Na/K ratio and the correction of cation and Na/K values for varying flow rates were also included. Two separate studies were instituted, the first concerned with relatively minimal stimulation and a low rate of flow, while the second experiment, carried out with more vigorous stimulation, involved analyses on samples collected at a higher rate of flow.

A review of the pertinent literature[1-13] revealed a potassium range of 5.63 to 30.13 mEq./L., while the sodium range extended from 3.30 to 89.15 mEq./L. Since many experimental variations were included in the past studies it seemed desirable to accomplish studies under rigidly controlled conditions on a sample of subjects sufficient in number to provide physiologic base lines.

PROCEDURE

All participants had undergone complete physical evaluation within a few days prior to testing and each had been found physically fit. The state of oral health was not a consideration in this primary study on these otherwise healthy subjects. Dietary factors, times of arising and retiring, and environmental conditions were virtually identical in all instances.

In the first experiment (1-RBS) samples were collected from 402 males and 60 females between 17 and 25 years of age under the stimulation of one small (size 18) rubber band. During the second experiment (3-RBS) 540 males and 116 females between 17 and 30 years of age were sampled under the stimulation of 3 large (size 32) rubber bands. All samples were collected at 9:00 A.M. and the duration of stimulation was, in all instances, exactly 5 minutes.

Subjects were instructed to clear their mouths by swallowing and each was then directed to chew vigorously on the chemically clean eliciting agent during the collection period. Saliva was expectorated into graduated centrifuge tubes, the specimens were immediately refrigerated, and dilution in preparation for analytical procedures was begun within 1 hour. Specimens containing grossly detectable blood were discarded.

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Analyses for sodium and potassium were carried out by internal standard flame photometric procedures,* 250 ppm of lithium being present both in controls and test dilutions. A suitable dilution of saliva was employed to produce a test sample that gave good reproducibility for both sodium and potassium.

Throughout the text and within the tables presenting means and standard deviations, unless otherwise indicated in table legends, the means represent calculations for the total number of subjects for each sex. All references in the text to standard deviations refer to the total standard deviation, the symbol therefor being $S.D._t$, which includes both the variation within the age groups and the added variation attributable to differences in age where such differences exist. The standard deviation value included in the tables as $S.D._w$ refers to the average standard deviation within age groups. This latter standard deviation value does not appear in the tables for the three rubber band study since no variations attributable to age were noted under this form of stimulation. In the 1-RBS portion, correlation coefficients, when calculated, were found for males only, since the sample size was relatively small for females.

RESULTS

One Rubber Band Study.—

The majority of the subjects, 443, were between 17 and 20 years of age, only 19 persons, 17 males and 2 females, not falling within these limits. These 17 males were between 21 and 25 years of age while the 2 females were 21 years old. The analyses of variance included only the 17- to 19-year-old males and the 18-year-old females.

TABLE I
DISTRIBUTION OF MEANS FOR MALES (ONE RUBBER BAND STUDY)

AGE	NUMBER OF SUBJECTS	VOLUME (ML./5 MIN.)	SODIUM (mEq./L.)	POTASSIUM (mEq./L.)	NA/K RATIO
17	149	4.17	12.11	24.65	0.51
18	146	4.94	13.32	24.13	0.57
19	68	4.51	14.75	24.64	0.62
20	22	5.43	15.08	23.47	0.68
21	10	5.05	18.36	22.98	0.83
22	5	4.30	17.99	24.96	0.74
25	1	3.20	20.50	22.45	0.91
N	402				
$\bar{X}$		4.60	13.42	24.35	0.57
$S.D._w$		2.43	7.06	3.48	0.34
$S.D._t$		2.44	7.16	3.48	0.34

$\bar{X}$ = mean for total number of subjects for each type of stimulation.
$S.D._w$ = average standard deviation within age groups.
$S.D._t$ = total standard deviation; this includes variation within age groups plus the added variation attributable to differences in age.

Volume: Rate of flow was found to increase significantly with age for males between 17 and 20 years of age (Table I). Females exhibited a tendency toward an increased rate of flow with age; however, this was not conclusive because the sample sizes were small except in the 18-year-old group (Table II).

*Baird Associates flame photometer.

TABLE II
DISTRIBUTION OF MEANS FOR FEMALES (ONE RUBBER BAND STUDY)

AGE	NUMBER OF SUBJECTS	VOLUME (ML./5 MIN.)	SODIUM (mEq./L.)	POTASSIUM (mEq./L.)	NA/K RATIO
18	48	2.01	13.43	25.17	0.54
19	8	2.10	12.94	25.52	0.53
20	2	3.20	12.90	24.93	0.52
21	2	7.00	18.05	20.05	1.02
N	60				
$\bar{X}$		2.23	13.50	25.04	0.55
S.D.$_w$		1.13	6.05	4.04	0.27
S.D.$_t$		1.43	5.95	4.05	0.27

$\bar{X}$ = mean for total number of subjects for each type of stimulation.
S.D.$_w$ = average standard deviation within age groups.
S.D.$_t$ = total standard deviation; this includes variation within age groups plus the added variation attributable to differences in age.

Flow rates for males and females differed markedly. The mean 5-minute volume for males was 4.60 ml. (S.D., 2.44), while females produced an average of only 2.23 ml. (S.D., 1.43). The flow rate data were markedly skewed. The range extended from 0.7 to 14.0 ml. for the 5-minute period.

Potassium: No statistically significant differences in potassium levels were found associated with age or sex. The distribution of potassium means is outlined in Tables I and II. The correlation coefficient between potassium and volume for males was estimated to be $r = -0.41$ ($P<.01$); that is, as the volume increased, the amount of potassium per unit volume tended to decrease. The potassium mean for males was found to be 24.35 mEq./L. (S.D., 3.48) while the mean for females was 25.04 mEq./L. (S.D., 4.05). Potassium, in original units, was approximately normally distributed.

Sodium: No statistically significant difference was found in sodium levels between males and females. Sodium was found to increase with age for males in the 17- to 20-year age groups (Table I). There was an indication that this was also true for females (Table II). A significant positive correlation, r estimated to be 0.27 ($P<.01$), was found between sodium levels and rates of flow for males; as the volume increased, the amount of sodium per unit volume tended to increase. The sodium mean for males was 13.42 mEq./L. (S.D., 7.16), and that for females was 13.50 mEq./L. (S.D., 5.95). As with volume, the distribution of sodium, in mEq./L., was found to be markedly skewed.

Na/K: The Na/K ratio was found to exhibit a pattern similar to that for sodium. No significant difference was found between the sexes but the ratio increased with age for males (Tables I and II). The male mean was 0.57 (S.D., 0.34) and that for females was found to be 0.55 (S.D., 0.27). The correlation coefficient between Na/K and volume, for males, was estimated to be $r = 0.42$.

Three Rubber Band Study.—

The analyses of variance did not include certain age groups for which the sample sizes were very small. For males, the 538 subjects in age groups 17 through 22 were included, and for females, the 102 participants in age groups 18 and 19 were included.

Volume: No significant flow rate differences associated with age were found for males or for females (Tables III and IV). There was a statistically significant difference attributable to sex. The volume mean for males was 8.05 ml. (S.D., 2.78) while that for females was 4.30 ml. (S.D., 2.18). The marked skew found in the volume data in the first experiment was considerably reduced under this increased stimulation.

TABLE III
DISTRIBUTION OF MEANS FOR MALES (THREE RUBBER BAND STUDY)

AGE	NUMBER OF SUBJECTS	VOLUME (ML./5 MIN.)	SODIUM (mEq./L.)	POTASSIUM (mEq./L.)	NA/K RATIO
17	168	8.08	19.97	22.34	0.92
18	203	8.03	21.02	22.23	0.97
19	101	8.38	21.46	22.11	1.00
20	44	7.96	22.00	22.72	1.02
21	13	6.02	20.08	23.33	0.89
22	0	7.72	23.98	22.50	1.10
23	2	6.60	10.18	23.69	0.43
N	540				
$\bar{X}$		8.05	20.84	22.32	0.96
S.D.$_t$		2.78	9.13	2.93	0.47

$\bar{X}$ = mean for total number of subjects for each type of stimulation.
S.D.$_t$ = total standard deviation; this includes variation within age groups plus the added variation attributable to differences in age.

TABLE IV
DISTRIBUTION OF MEANS FOR FEMALES (THREE RUBBER BAND STUDY)

AGE	NUMBER OF SUBJECTS	VOLUME (ML./5 MIN.)	SODIUM (mEq./L.)	POTASSIUM (mEq./L.)	NA/K RATIO
18	72	4.44	18.54	23.29	0.83
19	30	3.96	18.07	24.06	0.78
20	5	4.72	25.25	23.06	1.12
22-30	9	4.16	16.54	18.96	0.54
N	116				
$\bar{X}$		4.30	18.55	23.52	0.82
S.D.$_t$		2.18	8.02	3.50	0.41

$\bar{X}$ = mean for total number of subjects for each type of stimulation.
S.D.$_t$ = total standard deviation; this includes variation within age groups plus the added variation attributable to differences in age.

Potassium: For potassium, no significant differences due to age were found for males or for females (Tables III and IV). A significant difference ($P<.01$) was found between males and females. The male mean was 22.32 mEq./L. (S.D., 2.93); that for females was 23.52 mEq./L. (S.D., 3.50). The correlation coefficient between potassium and volume, for males, was estimated to be $r = -0.32$ ($P<.001$), while that for females was estimated as $r = -0.39$ ($P<.001$). Thus, potassium was negatively correlated with volume.

Sodium: Sodium levels did not differ significantly with age for males or for females (Tables III and IV). The mean value for males was 20.84 mEq./L. (S.D., 9.13) and that for females was 18.55 mEq./L. (S.D., 8.02). The difference between the means for males and females was statistically significant ($P<.01$). The correlation coefficient between sodium and volume was estimated to be $r = 0.28$ ($P<0.01$) for males, and $r = 0.16$ for females. The correlation coefficient for females was not statistically significant. Thus, for males, there

was an indication that sodium was positively correlated with volume. As with volume, the skew in the sodium data was much less than that noted under one rubber band stimulation.

Na/K: Analysis of the Na/K data revealed no significant differences associated with age for males or for females (Tables III and IV). The difference between the Na/K mean of 0.96 (S.D., 0.47), for males, and the mean of 0.82 (S.D., 0.41), for females, was found to be significant ($P<0.01$).

DISCUSSION

Volume: The 3 rubber band stimulation produced a 75.0 per cent increase in flow rate for males and a 92.8 per cent increase in flow rate for females. The increase in flow rate found with age in males in the 1-RBS study was not present in the 3-RBS portion. No satisfactory explanation based upon physiologic phenomena is offered to account for flow rate differences attributable to age in such a narrow age grouping.

Throughout the study a marked difference due to sex was noted in the rates of flow. Under one rubber band stimulation the mean flow rate for females was 48.5 per cent of the mean for males while, with increased stimulation, the flow rate mean for females was 53.5 per cent of the male flow rate mean. Psychologic influences may account for a portion of this significant difference between the sexes. It is possible that, since these samples were collected from subjects in groups rather than from individuals under conditions of privacy, the females were hesitant to expectorate in full view of the other participants. It was clearly evident that the cooperation of the male subjects was much more freely given than was that of the females in this study.

Potassium: A negative correlation between potassium and volume has been reported by, among others, Brown and Klotz,[4] Dreizen, Niedermeier, Reed, and Spies,[3, 8] Warming-Larsen, Hamburger, and Sprechler,[5] and deTraverse and Coquelet.[15] Other investigators,[9, 11, 17] after conducting animal or human studies, have concluded that no definite correlation exists between these 2 variables. In their studies, potassium was relatively independent of flow rates. Throughout the present study a negative correlation was found between potassium and volume.

When it was hypothesized that the potassium concentration would level off and not decrease further at a certain volume mean, volume observations were ranked, volume intervals were designated, and potassium means for males were calculated for these volume intervals (Table V). Under each form of stimulation there was an indication that the potassium concentration did level off, but in neither instance was it conclusive.

Sodium: Heidenhain's[17] law, formulated in 1878 as a result of experimentation on saliva from the submaxillary glands of dogs, pointed out that the total percentage of inorganic salts in saliva became greater as the rate of secretion was increased. Further experiments on animals[16] established that sodium consistently increased along with the secretion rate. Other investigations,[3, 4, 5, 8] carried out on human subjects, resulted in unanimous agreement that there was

a positive correlation between sodium levels and salivary flow rates. The findings of the present study are in accord with this conclusion with the exception of the correlation of sodium and volume found for females in the 3 rubber band study. In this instance, although a positive correlation coefficient was found, it was not statistically significant. No highly significant effects of rate of flow on the values of sodium or potassium could be found by White, Entmacher, Rubin, and Leiter.[9] These same investigators found, however, that subcutaneous doses of 6.0 mg. of pilocarpine lowered the salivary concentration of potassium by 3.97 mEq./L. and increased the sodium levels by 4.11 mEq./L. Unfortunately, no flow rate studies were carried out on these subjects, but, since the accelerative effect of pilocarpine on salivary flow rate is so well defined, these stated changes in the sodium and potassium levels would seem to discount the earlier conclusions drawn by White and his associates.

TABLE V
POTASSIUM MEANS ASSOCIATED WITH RANKED VOLUME INTERVALS

ONE RUBBER BAND			THREE RUBBER BANDS		
FLOW RATE (ML./5 MIN.)	NUMBER OF SUBJECTS	POTASSIUM MEANS (mEq./L.)	FLOW RATE (ML./5 MIN.)	NUMBER OF SUBJECTS	POTASSIUM MEANS (mEq./L.)
0.7- 1.5	22	26.41	1.7- 3.4	24	24.10
1.6- 2.0	22	26.02	3.5- 4.3	23	24.49
2.1- 2.4	28	25.99	4.4- 4.9	21	23.50
2.5- 2.7	27	25.85	5.0- 5.3	22	23.92
2.8- 3.0	26	25.08	5.4- 5.6	26	23.32
3.1- 3.4	23	24.93	5.7- 6.2	25	23.04
3.5- 3.7	27	26.25	6.3- 6.5	29	21.93
3.8- 3.9	22	24.58	6.6- 6.9	23	21.94
4.0- 4.3	24	24.16	7.0- 7.2	20	22.74
4.4- 4.7	27	24.77	7.3- 7.5	27	22.43
4.8- 5.1	24	24.06	7.6- 7.9	23	22.71
5.2- 5.7	23	23.44	8.0- 8.1	24	22.02
5.8- 6.4	22	22.88	8.2- 8.5	26	22.26
6.5- 6.9	21	22.23	8.6- 8.9	20	22.54
7.0- 8.0	20	22.14	9.0- 9.2	24	21.57
8.1- 9.3	22	21.49	9.3- 9.5	23	22.04
9.4-14.0	22	22.00	9.6- 9.9	24	22.17
			10.0-10.3	26	21.73
			10.4-10.7	23	21.20
			10.8-11.2	22	21.50
			11.3-12.1	23	19.98
			12.2-13.3	21	21.50
			13.4-19.5	20	20.48

The finding that there was no influence of sex on sodium levels in the 1-RBS experiment is in agreement with the observations of Grad.[11] In the 3-RBS study, however, the difference in sodium concentrations between the sexes was found to be significant ($P<0.01$). Under the lesser stimulus, sodium was found to increase with age in males and there was some indication that this was true for females. In the 3-RBS portion, sodium levels did not differ significantly with age for males or for females. It is of interest that, in the 1-RBS study, even with the male volume mean being more than double that for females, the sodium means for both sexes were approximately equal.

Na/K: Under one rubber band stimulation the results for the Na/K ratio were similar to the sodium findings. For sodium, a significant sex difference did not exist, but a significant increase with age was found for this variable. In the potassium analysis no significant differences due to age or sex were noted. Consequently, the results of the ratio of these two variables, Na/K, indicated that, like sodium, Na/K levels increased with age, and no significant difference between the sexes was found. Analysis of the data on the Na/K ratio (3-RBS) revealed no significant differences associated with age for males or for females but the difference between the sexes was significant.

Correction for Varying Rates of Flow.—

In the collection of stimulated saliva samples, only one of the two variables, time of stimulation or total flow, can be controlled in a given experiment. Rather than directing the subjects to produce samples of a predetermined size, it seems more practical, in collecting large numbers of specimens, to control the duration of stimulation to a precise time period, thus allowing a relatively wide variation in flow rates. Following this procedure, it would then be desirable that formulas be derived to correct for these flow rate variations and that a method and time of stimulation be selected such that the observed volumes would be in a range for which the derived formulas were valid.

As a result of experimentation on saliva samples obtained from 33 subjects, Prader, Gautier, Gautier, Naf, Semer, and Rothchild[14] derived formulas for the correction of sodium and Na/K values. These investigators found a positive correlation between flow rates and sodium, but they found no correlation between volume and potassium, in contrast to the negative correlation found in the present study. Consequently, in deriving their formulas they adjusted only their sodium and Na/K values for varying flow rates. Thus, they offered the following correction formulas to adjust the flow rates of all subjects to 2 ml. per minute:

$$Na_c = Na + (10 - \text{vol./5 min.})\ 1.6$$
$$(Na/K)_c = Na/K + (10 - \text{vol./5 min.})\ 0.1.$$

When the sodium and Na/K values of the 1-RBS study were thus corrected and analyzed, the results were similar to those for sodium and for Na/K (Tables VI and VII). A statistically significant difference attributable to sex was evident in the Na_c data. The Na_c mean for males was 22.09 mEq./L. (S.D., 6.86), while that for females was found to be 25.96 mEq./L. (S.D., 6.51). Since no significant difference due to sex was noted for sodium, it was evident that this apparent difference between the sexes in Na_c had been introduced by the use of the formulas. The $(Na/K)_c$ mean for males was 1.11 (S.D., 0.32), and that for females was 1.33 (S.D., 0.29). A significant increase in $(Na/K)_c$ with increasing age was found, this being the result of the increase in sodium levels with increasing age. The distinct difference in $(Na/K)_c$ between the sexes was due to the significant volume differences also noted between males and females.

In the 3 rubber band study the Na_c mean for males was 23.96 mEq./L. (S.D., 8.98) and that for females was 27.67 mEq./L. (S.D., 8.21). No significant differences due to age were found for males or for females but the difference between the sexes was significant ($P<0.01$). The $(Na/K)_c$ mean was 1.16 (S.D., 0.46) for males; 1.39 (S.D., 0.41) for females. A summary of these results is presented in Tables VI and VII. Age had no significant effect on $(Na/K)_c$ levels for either sex but there was a significant difference ($P<0.01$) between the values for males and for females.

TABLE VI
UNCORRECTED AND CORRECTED MEANS FOR SODIUM, POTASSIUM, AND THE Na/K RATIO

VARIABLE	ONE RUBBER BAND (MEANS—402 MALES)	THREE RUBBER BANDS (MEANS—540 MALES)
Na	13.42	20.84
Na_c*	22.09	23.96
Na_c†	15.92	19.03
K	24.35	22.32
K_c†	23.40	23.01
Na/K	0.57	0.96
$(Na/K)_c$*	1.11	1.16
$(Na/K)_c$†	0.72	0.86

*Corrected by formulas of Prader, et al.[14]
†Corrected by formulas derived from the present study.

TABLE VII
UNCORRECTED AND CORRECTED MEANS FOR SODIUM AND THE Na/K RATIO

VARIABLE	ONE RUBBER BAND (MEANS—60 FEMALES)	THREE RUBBER BANDS (MEANS—116 FEMALES)
Na	13.50	18.55
Na_c*	25.96	27.67
Na/K	0.55	0.82
$(Na/K)_c$*	1.33	1.39

*Corrected by formulas of Prader, et al.[14]

When the uncorrected and corrected data for sodium and Na/K in the one rubber band study were compared with those from the three rubber band experiment (Tables VI and VII), it was found that the pronounced differences in the uncorrected values between the studies were considerably reduced by the application of Prader's formulas. For males, the difference between sodium values in the 2 studies was 7.42 mEq./L., while the difference between Na_c values in the 2 was only 1.87 mEq./L. For females, a difference of 5.05 mEq./L. in sodium values was likewise reduced to 1.71 mEq./L. in the Na_c data. The differences in the uncorrected values of the ratio of the 2 cations were similarly reduced when correction formulas were used. For males, the difference between the Na/K means of the 2 studies (0.39) was reduced by correction to a $(Na/K)_c$ difference of only 0.05. For females, a Na/K difference of 0.27 was reduced to 0.06.

From the data of the present study (1-RBS vs 3-RBS) regression equations were derived correcting sodium, potassium, and Na/K values for varying flow rates. Regression coefficients of 1.248, –0.477, and 0.073, respectively, resulted.

Based upon the volume data and the number of subjects, rates of flow were corrected to 6.6 ml./5 min., the over-all mean for the 2 experiments. The following formulas resulted:

$$Na_c = Na + (6.6 - \text{vol./5 min.})\ 1.248$$
$$K_c = K - (6.6 - \text{vol./5 min.})\ 0.477$$
$$(Na/K)_c = Na/K + (6.6 - \text{vol./5 min.})\ 0.073.$$

The applications of these formulas to the male means for sodium, potassium, and Na/K produced Na_c, K_c, and $(Na/K)_c$ values that reduced the differences between the means for the two studies (Table VI). The difference in sodium values of 7.42 mEq./L. between the studies was reduced to 3.11 mEq./L.; the difference in potassium values of 2.03 mEq./L. was decreased to 0.39 mEq./L., and the Na/K difference of 0.39 became a $(Na/K)_c$ difference of 0.14.

SUMMARY

When a comparison was made between the results of salivary analyses on 402 males and 60 females stimulated by one small rubber band and 540 males and 116 females under three large rubber band stimulation, the following observations could be made:

1. Three rubber band stimulation produced a 75.0 per cent increase in flow rate for males and a 92.8 per cent increase for females. Flow rate increased with age in males between 17 and 20 years old under 1-RBS stimulation.

A significant volume difference attributable to sex was present in both studies. In the 1-RBS portion, the mean flow rate for females was 48.5 per cent of the mean for males while, under a 3-RBS stimulation, the female flow rate mean was 53.4 per cent of that found for males. Psychologic influences are offered as a partial explanation for this volume difference.

2. Potassium levels were not influenced by age under either form of stimulation. Under 3-RBS stimulation the difference attributable to sex was significant. Potassium and volume were found to be negatively correlated in both studies.

3. Sodium was found to increase with age in males between 17 and 20 years of age under 1-RBS stimulation. A significant difference in sodium levels was found between the sexes under 3-RBS stimulation. Sodium and volume were found to be positively correlated under each stimulus. This correlation was statistically significant in all instances except for females in the 3-RBS study.

4. The Na/K ratio (1-RBS) increased with age for males and no significant difference was found between the sexes. Under 3-RBS stimulation, no significant differences in Na/K with age were found, but the difference between the sexes was significant.

5. Correction formulas derived by Prader and his co-workers[14] were applied in an attempt to adjust the sodium and Na/K values for varying rates of flow. The differences in the resulting Na_c and $(Na/K)_c$ values between the studies were much smaller than the differences in the Na and Na/K values. These investigators did not derive formulas to correct the potassium values.

6. From the data of the present study, formulas were derived which corrected flow rates for the male participants to 6.6 ml./5 min. Differences in resulting Na_c, K_c and $(Na/K)_c$ values were small when the 2 studies were compared. These 3 formulas are offered as a means of reducing the distinct differences in Na, K, and Na/K mean values that are found associated with variations in means for rate of flow. Expressions based upon these corrected values would seem to be much more meaningful than statements based upon the uncorrected concentrations but it is to be emphasized that these statements are, at present, applicable only to mean values for large groups of subjects.

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SALIVARY SODIUM, POTASSIUM, AND CHLORIDE LEVELS IN SUBJECTS CLASSIFIED AS TO DENTAL CARIES EXPERIENCE

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A RELIABLE index of dental caries activity based upon the chemical analysis of the saliva has long been a dental research goal. The inorganic constituents of saliva have received considerable attention in this regard but, at the present time, controversial conclusions preclude the selection of any single component or group of components as an indicator of dental pathosis. A comparison of past studies indicates that much of the disagreement is the result of the marked variation in experimental procedures employed by the different investigators.

The present investigation was designed to determine the sodium, potassium, and chloride levels of mixed saliva collected from subjects whose dental status was rigidly classified. Concurrent studies have been and are being completed[1-3] in an effort to establish, under identical experimental conditions, physiologic base lines for these three variables without regard for oral pathology.

PROCEDURE

Each subject was given a thorough dental examination (illumination, mouth mirror, explorer) and only those persons with at least 20 teeth present were considered for this study. Bite-wing radiographs supplemented the clinical examination. All examinations and radiographic interpretations were carried out by the same individual. Subjects presenting any evidence of periodontal involvement, regardless of degree, were excluded from this study.

Three classifications of oral status were designated. From among the subjects considered for the study, only those whose oral condition was described by one of the 3 categories presented below were included.

1. *Carious*—at least 20 teeth present; at least 20 surfaces definitely involved by dental caries; no restorations of any type present.
2. *Resistant*—at least 20 teeth present; no evidence of dental caries; no restorations of any type present.
3. *Restored*—at least 20 teeth present; at least 20 surfaces restored with silver amalgam; no evidence of dental caries.

All calculations as to the number of surfaces involved by caries or restored with silver amalgam were based upon a modification of the Bodecker[4] index.

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All teeth were considered to present at least 5 surfaces. Based upon areas of caries susceptibility, one additional surface value was added to the following: occlusal surfaces of all maxillary molars, lingual surfaces of maxillary first and second molars, occlusal surfaces of lower premolars, and buccal surfaces of all lower molars. Thus, a total of 180 surfaces could be tabulated on 32 fully erupted permanent teeth.

All subjects had recently undergone thorough medical examination and each was found to be physically fit. Times of arising and retiring, types of food and drink ingested, and environmental factors were virtually the same for all subjects. Subjects varied in age from 17 to 23 years, with a mean age of 18.5 years for the entire group. The 17- and 18-year-old groups included 60 per cent of the participants while only 12 per cent were over 20 years of age.

Utilizing no exogenous salivary stimuli, whole saliva samples were collected from 537 male subjects. All samples were collected during the period extending from 8:00 A.M. to 10:30 A.M. The collection interval for each individual was precisely 5 minutes, during which time the accumulated saliva was collected into a graduated centrifuge tube. Samples which contained grossly detectable blood were discarded. The test specimens were refrigerated immediately and analyses were begun at the completion of the examination period.

Volume readings were taken directly from the graduated centrifuge tubes. Sodium and potassium concentrations were determined by flame photometry (Beckman DU; flame attachment; photomultiplier; spectral energy recording adapter) utilizing a hydrogen-oxygen flame. Chloride levels were determined by a modification of the method of Sendroy.[5] The salivary proteins were first precipitated with phosphoric-tungstic acid. The mixture was then shaken with an excess of solid silver iodate and centrifuged. After the addition of sodium iodide to a portion of the supernate, the resultant free iodine was titrated with 0.02308 N sodium thiosulfate.

The data obtained from these samples were analyzed to determine if significant differences existed in the levels of volume, sodium, potassium, or chloride for the 3 subject groupings. Since the means and standard deviations were calculated for relatively large groups of subjects, a second decimal is given. This does not necessarily imply laboratory accuracy of this degree.

RESULTS

The mean number of teeth for all subjects was 28.2. In the "Restored" subjects the mean value for filled surfaces was 40.6 (range—20 to 80), while, for the "Carious" group, the mean value for carious surfaces was 48.8 (range—21 to 146). Since none of the "Carious" subjects presented dental restorations of any type and since the "Restored" participants presented no carious lesions, the question of the validity of indices which consider both conditions in the same mouth is of no significance in the present study.

Volume.—No significant differences were found in the volume levels (ml./5 min.) for the 3 subject groups (Table I). The volume mean for all 537

subjects was 5.42 ml., with group means of 5.46, 5.55, and 5.23 ml. for the "Carious," "Resistant," and "Restored" groups, respectively.

Potassium.—No significant differences were noted in potassium levels for the 3 groups (Table I). The potassium mean for all subjects was 22.70 mEq./L., while the mean for the "Carious" group was 22.51 mEq./L., that for the "Resistant" group was 22.89 mEq./L., and that for the "Restored" subjects was 22.63 mEq./L.

Sodium.—Significant differences at the 5 per cent level were found in sodium values between certain groups (Table I). The sodium mean for all participants was 10.13 mEq./L. The mean sodium level for the "Resistant" group (9.58 mEq./L.) was found to be significantly lower ($P<.05$) than the mean sodium level for the "Carious" group (10.41 mEq./L.) or the "Restored" group (10.57 mEq./L.). There was no significant difference between the "Carious" and "Restored" groups. For the subject groups which were significantly different, 95 per cent confidence limits for the difference between the means were estimated to be:

"Carious" and "Resistant"	0.002-1.658
"Resistant" and "Restored"	0.186-1.790

Thus, it could be said with 95 per cent confidence that the interval from 0.002 to 1.658 contained the true mean difference between the "Carious" and "Resistant" groups. A similar statement concerning the interval from 0.186 to 1.790 could be made in regard to the true mean difference between the "Carious" and "Restored" categories.

TABLE I
DISTRIBUTION OF VOLUME, POTASSIUM, SODIUM, AND CHLORIDE MEANS

SUBJECT GROUPS	NUMBER OF SUBJECTS	VOLUME MEANS (ML./5 MIN.)	POTASSIUM MEANS (MEQ./L.)	SODIUM MEANS (MEQ./L.)	CHLORIDE MEANS (MEQ./L.)
Carious	153	5.46	22.51	10.41	23.63
Resistant	213	5.55	22.89	9.58	22.52
Restored	171	5.23	22.63	10.57	23.73
$\overline{N}$	537				
$\overline{X}$		5.42	22.70	10.13	23.22
$S.D._w$		1.85	3.79	3.99	4.42
$S.D._t$		1.85	3.78	4.00	4.45

$\overline{X}$ = mean for 537 male subjects.
$S.D._w$ = average standard deviation within subject groups.
$S.D._t$ = total standard deviation; this includes variation within each subject grouping plus the added variation, if any, attributable to differences between subject groups.

Chloride.—Significant differences were also found between the chloride means for certain of the 3 classifications of subjects (Table I). A mean chloride value of 23.22 mEq./L. was found for all subjects. The mean for the "Resistant" group (22.52 mEq./L.) was significantly lower ($P\simeq0.018$) than that found for the "Carious" participants (23.63 mEq/L.). The mean for the "Resistant" group was significantly lower ($P\simeq0.008$) than that for the "Restored" subjects (23.73 mEq./L.). No significant difference was found

between the "Carious" and "Restored" groups. Confidence limits (95 per cent) for the differences between the chloride means for the groups which were significantly different were estimated to be:

"Carious and "Resistant"	0.188-2.024
"Resistant" and "Restored"	0.317-2.095

DISCUSSION

Before any valid discussion of the results of salivary analyses can be undertaken, it is imperative that the type of saliva under study be definitely established. Many reports in the literature erroneously refer to saliva samples as "resting" or "nonstimulated" simply because the subjects did not chew some exogenous stimulation agent during the collection period. Since the presentation of a collection tube and the explanation of the collection technic provides a psychic stimulus and since subjects are prone to stimulate the flow of saliva by oral movements, the omission of an exogenous eliciting agent represents only a change in the degree of stimulation. Since no eliciting agent was employed, the saliva collected in the present study represents saliva accumulated in the oral cavity under conditions of minimal stimulation.

Volume.—There is disagreement in the literature concerning the possible relationship between salivary flow rates and the incidence of dental caries. It has been found that children and adolescents who have markedly decreased salivary flow rates also have early and rampant dental decay and that a lack of salivary flow predisposes to caries in experimental animals,[6] Miller[7] observed a negative correlation between salivary flow rates and caries attack rates, while Trimble, Etherington and Losch[8] after studying 57 students, reported that subjects with lower flow rates had a higher caries incidence. Becks, Wainwright and Young[9] found practically identical average rates of flow when their caries-free and caries-active groups were compared. Under the conditions of the present study, no significant volume differences were noted for the 3 experimental groups.

Potassium.—White and his co-workers[10] noted a high salivary potassium concentration associated with a high caries rate and a low content associated with freedom from caries. On the other hand, Wach[11] compared 10 caries-immune subjects to 14 caries-active ones and found that salivary potassium levels were higher in those persons free from caries. The results of the present study (537 subjects) are not in accord with either of these findings since no significant difference in potassium values existed between the 3 groups of subjects.

Sodium.—White[10] concluded that there was no definite relationship between caries activity and the salivary sodium concentration. However, the findings of the present study indicate that sodium levels of saliva from both the "Carious" and "Restored" subjects were significantly higher than the sodium levels of the subjects with neither caries nor restorations.

Chloride.—Chlorides are known to enhance amylase activity. Since it has been reported[12-15] that the amylase activity of saliva from caries-active subjects

is significantly higher than that from subjects free of caries activity, salivary chloride levels have received considerable attention. However, neither Hubbell[16] nor Roskin[17] were able to find a significant relationship between chloride levels and caries activity. On the other hand, Anders[18] found that chloride levels were apparently related to caries activity. He studied 251 children, divided into 7 categories based upon caries incidence during the preceding 12 months. The chloride mean for his caries-free subjects was 12.59 mEq./L. as compared to 18.35 mEq./L. for the extensive, active rapid decay group.

Although the chloride means found in the present study are considerably higher than those reported by Anders,[18] both studies indicate that whole saliva chloride values are significantly lower in caries-resistant subjects than in caries-active subjects.

SUMMARY

Using no exogenous salivary stimuli, saliva samples were collected from 537 males, each of whom had been placed by clinical and radiographic examination into one of 3 restrictive oral status categories (''Carious,'' ''Resistant,'' or ''Restored''). After the completion of analytical procedures, the following results were evident.

1. No significant differences in volume (ml./5 min.) levels were noted between the 3 groups of subjects.

2. The mean potassium levels were not significantly different for the 3 categories.

3. The mean sodium level for the ''Resistant'' group was significantly lower ($P<.05$) than the mean sodium values for the other 2 groups. No significant difference was found between the ''Carious'' and ''Restored'' categories.

4. The chloride mean value for the ''Resistant'' group was significantly lower ($P\simeq0.018$) than the mean level for the ''Carious'' subjects. A similar but even more significant ($P\simeq0.008$) difference was found between the chloride means of the ''Resistant'' and ''Restored'' subjects.

5. Although significant differences were found for both the salivary sodium and chloride levels when ''Resistant'' and ''Carious'' subjects were compared, no significant differences were noted between the ''Carious'' and ''Restored'' groups.

6. The findings of the present study indicate that the predictability of an individual's dental status based upon salivary volume, sodium, potassium, and chloride findings would be, at best, extremely low. Although significant differences were found when the means for the 3 groups of subjects were compared, there was an extensive overlap in values for individuals throughout the 3 groups.

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THE RELATIONSHIP OF FOOD CONSUMPTION AND SALIVARY FLOW TO THE INCIDENCE OF DENTAL CARIES IN THE RAT

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MUHLER[1] has shown that the frequency of food intake, as well as the amount of food ingested, is important in determining the incidence of experimental dental caries in the rat. In a previous study the importance of food impaction in the sulci of the molars and the effect of salivary flow on the production of dental caries in the rat was studied.[2] This latter study suggested that the absence of saliva was more important in extending a previously initiated carious lesion than in initiating a new one. The purpose of this present study was to determine the importance of food consumption on the initiation and extension of dental caries in the rat in the presence of reduced salivary flow.

EXPERIMENTAL

A total of 120 weanling albino rats (Sprague-Dawley strain) were equally divided into 4 experimental groups on the basis of initial body weight and sex. The animals in Group 1 had their major salivary glands surgically removed and received food ad libitum. Group 2 was composed of animals whose daily food consumption was restricted to 60 per cent of that amount consumed by unoperated control rats (Group 4) which received food ad libitum. Group 3 had its major salivary glands removed and also had its daily food consumption restricted to the same level as the animals in Group 2. Group 4 was composed of unoperated rats whose individual daily food consumption was unrestricted but measured daily. In each instance, the food consumption of males was compared only to males and females only to females. All animals were housed individually in raised metal cages in an air-conditioned room for the experimental period of 140 days. All of the animals received fluorine-low water (F = 0.05 μg/ml.) and a stock corn cariogenic diet.[2] At the end of the experimental period, the animals were sacrificed by ether and the heads removed for dental caries evaluation by methods previously described.[3]

RESULTS AND DISCUSSION

The effects of diet restriction and desalivation on the dental caries incidence are shown in Table I. Removal of the major salivary glands produced a significant increase in the caries incidence, while the restriction of food intake to 60 per cent of the amount normally consumed by the unrestricted control

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group resulted in a highly significant ($p = < 0.001$) reduction in caries. This latter finding confirms the work of Muhler[1] in which a reduction of 75 per cent in the caries experience was noticed when rats were restricted to a 60 per cent dietary intake. The animals on the restricted diet also have a marked reduction in the extent of their carious lesions. The group which received a 60 per cent dietary allowance, and also had their salivary glands removed, showed a caries incidence similar to the control. The limiting of food intake in desalivated rats significantly reduced their caries incidence when compared to desalivated rats receiving the cariogenic diet ad libitum.

TABLE I
THE EFFECT OF DESALIVATION AND FOOD RESTRICTION ON DENTAL CARIES INCIDENCE IN THE RAT

GROUP	NO. ANIMALS	SEX	FINAL BODY WT. (GM.)	MEAN NO. LESIONS (SEXES COMBINED)	PER CENT CHANGE (%)	MOLARS AFFECTED
Restricted diet	14	Male	225	3.2	−63	2.1
	20	Female	166			
Desalivate	15	Male	311	12.1	+28	6.3
	15	Female	209			
Desalivate on restricted diet	13	Male	190	9.0	+ 2	5.5
	12	Female	141			
Control	12	Male	370	8.8	--	4.6
	10	Female	242			

The results of this study again demonstrate that the absence of saliva is associated with a significant increase in dental caries in the rat. Food restriction also significantly reduced dental caries—an observation which has previously been demonstrated. The question of which of the factors is most important in controlling the total dental caries incidence is partially answered by examining the data obtained when the two factors are combined. These data suggest that desalivation, even under conditions of food restriction, is a significant cariogenic factor. However, the importance of food restriction is still manifest since there is significantly less ($p = 0.01$) dental caries in the group which is desalivated and diet restricted than in the ad libitum fed desalivated group. These data fail to show convincing evidence in regard to the single effects of restricted salivary flow or food restriction on caries development, since it may well be that the combination of food restriction and salivary flow reduction retards the entire caries process and not simply the initiation or progression rates.

The reason for the pronounced caries reduction in the animals receiving the food restriction is not known and additional evidence is needed. It may partially be explained on the basis that caries development in the rat is dependent upon a coarse corn diet, and a reduction in the volume of food consumed concomitantly reduces the number of lesions. However, it is equally possible that, as a result of food restriction, the animals eat less frequently throughout the day and the teeth have less contact with the cariogenic influence of the diet.

The role of food ingestion and the dental caries incidence in the rat needs further clarification, especially since food restriction results in pronounced weight changes, and it is known that the pituitary[4] and thyroid[5, 6] glands are related to the caries incidence in the rat.

SUMMARY

The cariogenic influence of desalivation and the anticariogenic effect of food restriction has been demonstrated. A combination of influences resulting from restricting the food intake of desalivated rats suggests that the absence of saliva is a more pronounced cariogenic factor than diet restriction is an anticariogenic factor. However, these data are not convincing in regard to the individual effects of desalivation and food restriction, since the data obtained, when both factors are combined, indicate a decreased rate of caries formation and not a reduction in the incidence of caries.

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THE EFFECT OF PILOCARPINE IN SINGLE OR DIVIDED DAILY DOSES ON THE INCIDENCE OF DENTAL CARIES IN THE RAT

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PREVIOUS work by Muhler, Bixler, and Shafer[1] has shown that 2 mg. of pilocarpine administered daily by stomach tube resulted in significantly less dental caries in rats than when 12 mg. a day was received. The reason why higher doses failed to reduce dental caries is not known, but could be related to the fact that at this level the drug has an unphysiologic action on the salivary glands. It is also possible that this high level of pilocarpine depletes the stores of or decreases the production of some substance present in the salivary gland responsible for the anticariogenic effect which results from feeding desiccated thyroid. It seemed reasonable that a frequently administered low dose might be more anticariogenically effective than a single large dose. The purpose of this paper is to describe the results of administering a low dose of pilocarpine, known by previous work to reduce dental caries, in a single daily dose and in 3 divided daily doses. An evaluation is also made of pilocarpine when fed as a constituent of the diet. This is considered to be comparable to a continuous administration of the drug.

EXPERIMENTAL

A total of 160 weanling Sprague-Dawley strain rats were equally divided into 4 groups, according to sex and initial body weight, of which 125 animals finished the study (Table I). Groups 1 and 2 received 2 mg. of pilocarpine nitrate daily by stomach tube. Group 1 received the pilocarpine in a single dose and Group 2 received it divided into 3 doses, spaced approximately 4 to 5 hours apart. Group 3 received the pilocarpine as a constituent of the diet at such a level (0.134 mg. per kilogram) calculated to furnish 2 mg. a day per rat on the basis that each rat would eat approximately 15 grams of food daily. Group 4 received no pilocarpine and served as controls. All of the animals received the same stock corn cariogenic diet† and fluorine-low (F = 0.05 μg/ml.) drinking water ad libitum, and were housed in raised screen cages in an air-conditioned room. The duration of the experiment was 140 days, after which the animals were sacrificed by ether and the heads removed for dental caries evaluation by methods previously described.[2]

DATA AND DISCUSSION

The effect of pilocarpine on the incidence of dental caries in the rat is seen in Table I. These data indicate that the pilocarpine significantly reduced the dental caries experience regardless of whether it was administered once a day,

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†Composition of diet was as follows: yellow corn grits, 52.7%; ground yellow whole corn, 11.3%; powdered whole milk, 30%; alfalfa, 4.8%; iodized sodium chloride, 1%; and irradiated yeast, 0.2%.

3 times a day, or ingested ad libitum in the diet. There appears to be a tendency for more caries in the group receiving the pilocarpine 3 times a day than when received only once a day or when received ad libitum in the diet. The reason for this is not known and is difficult to explain on the basis of present evidence since it was suspected that if the salivary volume was increased 3 times a day there would be less caries than if it were increased only once a day, provided, of course, that increased volume is related inversely to the dental caries experience. Since previous data[1] have suggested that levels of pilocarpine above 2 mg. a day are not associated with a significant reduction in dental caries, it may be that increasing the amount or the frequency of administration alters the salivary constituents in such a manner as to inhibit the caries-reducing effects of the pilocarpine. However, none of these levels significantly alters the normal histologic picture of the submaxillary glands.[3] Apparently, any changes in salivary gland function are expressed on a molecular level. These results indicate that additional work on the effect of pilocarpine on salivary composition is needed before pilocarpine administration is suggested for the control of rampant caries in children.

TABLE I
A COMPARISON BETWEEN THE EFFECT OF PILOCARPINE ADMINISTERED BY STOMACH TUBE ONCE A DAY OR THREE TIMES A DAY ON THE DENTAL CARIES EXPERIENCE IN RATS

GROUP	NO. OF ANIMALS	SEX	BODY WEIGHT GAIN (GM.)	MEAN NO. OF DENTAL LESIONS (SEXES COMBINED)	P*
Once a day	17	Male	281	6.5	0.02
	13	Female	175		
Three times a day	16	Male	277	7.4	0.05
	16	Female	168		
In the diet	16	Male	295	6.0	0.01
	16	Female	165		
Control	18	Male	297	9.3	---
	14	Female	175		

*Significance calculated on the basis of the t test.

SUMMARY

The same amount of pilocarpine was given to rats either once a day, 3 times a day, or in the diet which was fed ad libitum and the dental caries experience compared to control animals receiving no pilocarpine. The data indicated that all 3 groups receiving the pilocarpine had a significant reduction in dental caries. The anticariogenic effect of pilocarpine does not seem to be related to frequency of ingestion, but instead may be related to differences in the chemical composition of the saliva following pilocarpine administration.

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THE SOLUBILITY OF INTACT DENTAL ENAMEL SURFACES TREATED WITH POTENTIAL ANTICARIOGENIC AGENTS

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PREVIOUS animal studies[1] in which highly concentrated fluoride solutions were used have shown the superiority of stannous fluoride over sodium fluoride as an inhibitor of acid solubility. The primary reason for the development of such technics as used in animal studies was to test the proposed antisolubility agents under chemical conditions which subsequently would be used clinically. It would be quite helpful to develop an in vitro screening technic which would evaluate fluoride solutions at concentrations used clinically, and which would accurately predict the clinical effectiveness of the compounds under investigation. This paper describes a technic using intact enamel surfaces for such a purpose.

EXPERIMENTAL

Throughout this investigation, human teeth were used and were stored immediately after extraction in fluorine-free water until used. Following the mounting of the teeth they were again stored in water, and at no time during the investigation were they allowed to become dehydrated.

The preparation of the teeth for determining their acid solubility was as follows: The root was removed about 1 mm. below the cemento-enamel junction. The whole tooth was completely covered with self-curing resin, except for a window comprising the entire labial surface of the incisors and canines and the buccal surface of the premolars and molars. Each tooth served as its autocontrol, so it was not necessary to assure oneself that an identical amount of the labial or buccal surface was exposed on different teeth.

Individual teeth were placed into a small beaker containing 10 ml. of 0.2 N acetic acid, adjusted to pH 4 with sodium hydroxide. The solution was stirred by means of a mechanical stirring apparatus for 20 minutes. Immediately following the first 20-minute stirring interval, the teeth were removed, washed with distilled water, blotted dry with filter paper, and immediately placed into a new beaker containing 10 ml. of fresh 0.2 N acetic acid. This procedure was repeated 4 times. The solutions of the first and second acid treatments were not analyzed, but those from the third and fourth acid treatments were independently analyzed for phosphorus, by the method of Fiske and Subbarow,[2] and served as the control solubility values. The teeth were thoroughly washed in distilled water and placed in a beaker containing 10 ml. of the protective solution and stirred for 20 minutes. Following thorough rinsing in distilled

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water again, the teeth were placed in a beaker with 10 ml. of 0.2 N acetic acid, pH 4, and stirred for an additional 20 minutes. Each of these solutions was independently analyzed for phosphorus. The degree of protection of the unknown compound against acid solubility was estimated by comparing the amount of phosphorus lost in the first decalcification following the protection treatment with the average of the phosphorus lost during the third and fourth control decalcifications.

DATA AND DISCUSSION

The results of this study clearly indicate the superiority of stannous fluoride among the enamel antisolubility agents tested (Table I). These data are of interest when compared with previous studies evaluating these same compounds using powdered enamel[3] or experimental animal dental caries studies,[4] which indicated the marked ineffectiveness of sodium oxalate and sodium-N-palmitoyl sarcosinate in inhibiting powdered enamel and rat dental caries.

TABLE I

A COMPARISON OF THE PROTECTION AGAINST ACID SOLUBILITY BY DIFFERENT COMPOUNDS USED TO TOPICALLY TREAT INTACT HUMAN ENAMEL SURFACES

COMPOUND	CONC. (%)	NO. OF TEETH	MEAN P REMOVED (μg) A	MEAN P REMOVED (μg) B	REDUCTION IN INTACT ENAMEL SOLUBILITY (%)
Stannous fluoride	0.1	6	30.3	2.5	92 ± 1
	1.0	6	26.3	1.5	94 ± 2
	2.0	6	27.9	1.7	94 ± 2
Stannous sulfate*	0.1	6	31.1	28.7	8 ± 5
	1.0	12	31.3	29.8	5 ± 2
	2.0	30	32.8	29.5	10 ± 3
Sodium fluoride	0.1	12	28.0	19.6	30 ± 4
	1.0	12	30.6	21.2	31 ± 4
	2.0	12	32.5	20.2	38 ± 8
Calcium zirconium fluoride	0.1	6	28.0	6.2	78 ± 3
	1.0	6	48.9	10.4	79 ± 2
	2.0	6	35.3	6.6	81 ± 1
Sodium oxalate	0.1	12	37.8	41.3	− 9 ± 8
	1.0	12	30.3	33.8	−12 ± 4
	2.0	12	27.6	36.3	−32 ± 6
Sodium dehydroacetate	0.1	12	32.8	33.2	− 1 ± 3
	1.0	6	28.0	30.5	− 9 ± 5
	2.0	12	37.0	42.2	−14 ± 7
Sodium-N-palmitoyl sarcosinate	0.1	12	28.9	25.4	12 ± 5
	1.0	12	36.0	32.1	11 ± 2
	2.0	12	26.3	24.0	9 ± 6

A—P liberated in acid before protective treatment.
B—P liberated after protective treatment in acid.
*This compound has the following formula: $SnSO_4 \cdot NaCl \cdot NaHSO_4$.

The effect of increasing the fluoride concentration from 0.1 per cent to 2 per cent appears to have little effect in further reducing the antisolubility effectiveness of stannous fluoride, sodium fluoride, or calcium zirconium fluoride, but increasing the concentration of sodium oxalate appears to increase the solubility of the enamel.

CONCLUSIONS

A technic in which the acid solubility of human teeth treated with compounds which modify the surface solubility has been presented. The results indicate a superiority for stannous fluoride over sodium fluoride and calcium zirconium fluoride. Sodium oxalate, sodium dehydroacetate, sodium-N-palmitoyl sarcosinate, and stannous sulfate were ineffective at the concentrations used in significantly inhibiting the acid solubility of the intact enamel surface.

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THE EFFECT OF A SINGLE TOPICAL APPLICATION OF STANNOUS FLUORIDE ON THE INCIDENCE OF DENTAL CARIES IN ADULTS

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GISH, Howell, and Muhler[1, 2] have reported that a single application of an 8 per cent solution of stannous fluoride applied once each year is significantly more effective than 4 applications of a 2 per cent solution of sodium fluoride applied every 3 years. This pronounced anticariogenic effect in children prompted us to test the effectiveness of a highly concentrated solution of stannous fluoride for the partial control of dental caries in adults.

EXPERIMENTAL

Approximately 500 Indiana University freshmen, 17 to 38 years of age, were divided according to previous dental caries experience[3] into 2 groups. Group I received a single application of distilled water, and Group II a single application of a 10 per cent unbuffered aqueous solution of stannous fluoride. Both groups received their respective treatments immediately following a thorough prophylaxis. The stannous fluoride solution was prepared and used immediately according to technics previously described.[4] Each adult received a 7-film bite-wing examination and clinical dental caries examination at the initial visit and again after a 12-month period. Similar precautions were taken in order to eliminate bias both in the examinations and recordings of the data as were taken in previous studies.[1, 4]

DATA AND DISCUSSION

The dental caries data from the water and stannous fluoride treated groups are seen in Table I. The single application of stannous fluoride reduced the caries increment by 24 and 16 per cent when evaluated by the DMFT and DMFS indices, respectively. When the results of this study are compared to those obtained in children when a single application of an 8 per cent stannous fluoride solution is used, the anticariogenic effectiveness of the stannous fluoride appears to be less. The reason for these differences are not known, but good evidence does suggest that the effectiveness of topical fluoride treatments in the adult dentition is markedly less effective than in children. Additional experimental investigations are needed to learn more about the chemical and physical reactions of fluorides with the enamel in children and adult teeth.

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TABLE I

THE EFFECT OF A SINGLE TOPICAL APPLICATION OF A 10 PER CENT UNBUFFERED AQUEOUS SOLUTION OF STANNOUS FLUORIDE ON CARIES REDUCTION IN ADULTS AFTER A 1-YEAR PERIOD

	GROUP		DENTAL CARIES REDUCTION (%)
	CONTROL	SnF_2	
Number of subjects	207	228	
DMFT increment	0.95	0.72	24 ($p = .04$)*
DMFS increment	2.98	2.50	16 ($p = .08$)
Proximals	2.03	1.50	26
Occlusals	0.64	0.57	11
Buccal-lingual	0.31	0.42	−35

*Probability based upon the t test.

SUMMARY

The effectiveness of a single topical application of a 10 per cent stannous fluoride solution was evaluated for its ability to reduce the dental caries experience in adults. One year following the topical treatment with stannous fluoride, there were 24 per cent less dental caries when evaluated by the DMFT index and 16 per cent less when the DMFS index was used when compared to a similar group receiving a single topical application of distilled water. These reductions were significant at the 0.04 and 0.08 level of confidence, respectively.

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THE EFFECT OF TOPICALLY APPLIED POTASSIUM FLUOROSTANNITE ON THE DENTAL CARIES EXPERIENCE IN CHILDREN

II. Results at the End of Two Years

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IN a previous publication, the effectiveness of a 4 per cent solution of potassium fluorostannite in reducing the dental caries experience in children at the end of 1 year was presented.[1] These data indicated 53.7 and 39.2 per cent less dental caries in the group receiving the potassium fluorostannite when compared to a group receiving distilled water, as evaluated by the DMFT and DMFS indices, respectively. Both of these differences were highly significant. The data presented in this paper, obtained 2 years following the initial treatments, are important in evaluating the duration of effectiveness of this compound.

EXPERIMENTAL

The design of this study has been described.[1] Essentially, it involves the initial treatment of 442 children, 6 through 16 years of age, with 4 applications of a 4 per cent aqueous solution of potassium fluorostannite. An identical number of children of the same past dental caries history and age range were treated with 4 applications of distilled water. The same dentist performed all the clinical examinations (C. W. G.).

RESULTS AND DISCUSSION

The results of the 4 applications of the potassium fluorostannite on reducing dental caries in children 2 years following the initial treatment are seen in Table I. These data demonstrate that a significant effect of the fluoride remained after 2 years. There was a 47.5 per cent reduction in dental caries as evaluated by the DMFT index, and a 38.5 per cent reduction when the DMFS index was used, both reductions being highly significant as indicated by their probabilities (Table I). When the teeth and surfaces extracted during the study period are not counted in the DMFT or DMFS indices, the reduction becomes 46 and 47 per cent, respectively, for the DMFT and DMFS indices. Thus, the result of having teeth extracted during the study period has an effect on the apparent over-all caries reduction.

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TABLE I

A COMPARISON OF CARIES INCREMENTS AFTER 2 YEARS IN TWO GROUPS OF CHILDREN, ONE OF WHICH RECEIVED FOUR APPLICATIONS OF A 4 PER CENT SOLUTION OF POTASSIUM FLUOROSTANNITE AND THE OTHER FOUR APPLICATIONS OF DISTILLED WATER

	GROUP		
	DISTILLED WATER	POTASSIUM FLUOROSTANNITE	REDUCTION (%)
Number of subjects	364	319	
DMFT increment	1.83	0.96	47.5 (p=0.00001)*
DMFS increment	4.42	2.72	38.5 (p=0.00006)
Proximal	2.27	1.47	35
Occlusal	1.27	0.68	46
Buccal-lingual	0.88	0.57	35

*Probability based upon "t" test.

After 2 years, there was no significant decrease in the effectiveness found at the end of 1 year. It is not possible to compare these data with the previously published clinical data of Howell and Muhler[2] in which stannous chlorofluoride was used, or with that of McLaren and Brown[3] where stannous fluoride was used, since all 3 would have to be compared in one study. However, in both studies referred to above, a significant reduction in effectiveness was repeated at the end of the 2-year period when compared to the 1-year results. These data, as well as others, have suggested the superiority of the "tin" fluorides over sodium fluoride. More work on related analogues is needed in an attempt to find even more effective anticariogenic agents.

SUMMARY

Two years following the initial topical treatment of children with a 4 per cent potassium fluorostannite solution there were 47.5 and 38.5 per cent less dental caries as evaluated by the DMFT and DMFS indices, respectively, when compared to a similar control group which received distilled water. These 2-year data indicate that there was no significant loss in effectiveness when compared to the 1-year results.

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THE EFFECT OF A DIET COMPOSED OF BARLEY AND FISH MEAL ON DENTAL CARIES IN RATS

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IN a previous study by Buttner, Cremer, and Hartung,[1] it was shown that a diet composed of barley and fish meal was highly cariogenic for the rat. The original composition of this diet was described by Cremer and Rozeik.[2] However, in the study of Buttner, Cremer, and Hartung,[1] the rats were salivariadenectomized, and it is felt that this operation results in such pronounced unphysiologic effects on the oral environment, that the evaluation of dietary factors under such conditions is not optimal. In the above report, it was also shown that a reduction in dental caries could be caused by supplementing the cariogenic diet with 5 or 10 per cent yeast. The anticariogenic effect produced by the yeast was suggested to be the result of the inorganic composition of the yeast since similar caries reductions resulted by adding ashed yeast to the cariogenic diet. Similar anticariogenic effects of yeast were shown in a study by Dalderup and Jansen,[3] and were confirmed in a part of the above-mentioned report[1] in non-salivariadenectomized rats receiving a purified diet which contained 63 per cent sucrose. The purpose of this report, then, is twofold: (1) to study the suggested cariogenic effect of a barley-fish meal diet in the control non-salivariadenectomized rat; and, (2) to receive further evidence concerning the anticariogenic effect of yeast.

EXPERIMENTAL

A total of 120 weanling Sprague-Dawley strain rats were divided equally into 4 groups according to sex and initial body weight of which 98 rats finished the experiment. The diets which the 4 groups received are shown in Table I.

TABLE I
COMPOSITION OF DIETS

DIETARY COMPONENTS	DIETS			
	I	II	III	IV
	(%)			
Ground barley	50	50	50	50
Sucrose	25	–	–	25
Fish meal	25	25	25	5
Vitamin test casein	–	–	15	–
Corn grits	–	25	–	20
Irradiated yeast	–	–	10	–

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The animals were housed in pairs in raised screen cages in an air-conditioned room. The duration of the study was 90 days, after which time the animals were sacrificed by ether and the heads removed and scored for dental caries as previously described.[4] All of the animals received the diets and fluoride low drinking water (F = 0.05 μg/ml.) ad libitum.

DATA AND DISCUSSION

The effect of the various diets on the dental caries experience in rats is seen in Table II. The animals receiving Diets I, II, and III had significantly less dental caries than those receiving Diet IV. Apparently Diet I, composed of 50 per cent barley, 25 per cent fish meal, and 25 per cent sucrose, is not as cariogenic in non-salivariadenectomized rats as a similar diet used by Buttner, Cremer, and Hartung[1] in animals without their major salivary glands. These data suggest that perhaps the severity of the physiologic change resulting from salivariadenectomy so overshadows the cariogenic influence of the diet, that such procedures are not reliable for dietary-dental caries studies. The data may also suggest, however, that the presence of fish meal in the diet may confer anticariogenic influences mediated by way of the salivary glands. At present, the reason for the difference between these data and those of others[1] is not known. However, the method of preparing dehydrated feeds can greatly influence their nutritional qualities with the result that all fish meals are not all equally nutritious. The fish meal used by Buttner, Cremer, and Hartung[1] could well have differed nutritionally in some important factors from those used in this study.

TABLE II
A COMPARISON OF THE CARIES SCORES IN RATS RECEIVING VARIOUS EXPERIMENTAL DIETS

GROUP	SEX	NO. OF ANIMALS	WEIGHT GAIN (GM.)	DENTAL CARIES EXPERIENCE (MEAN NO. OF LESIONS)		p*
I	Male	10	158	2.5 ± 0.6†	2.9‡	<0.01
	Female	14	125	3.2 ± 0.7		
II	Male	14	161	3.6 ± 0.9	3.5	<0.01
	Female	13	123	3.5 ± 1.0		
III	Male	8	95	1.9 ± 0.6	2.8	<0.01
	Female	12	79	3.5 ± 1.0		
IV	Male	13	171	7.3 ± 0.8	6.3	-
	Female	14	125	5.4 ± 0.8		

*Probability (based upon "t" test) estimated from dental lesions with sexes combined.
†Standard deviation.
‡Sexes combined.

The presence of sucrose does not seem to be associated with an increased dental caries experience in rats in this particular study, for the same amount of sucrose in Diet IV as used in Diet I results in significantly more dental caries. The presence of sucrose alone in a diet may not be an adequate influence to initiate dental caries in the absence of other initiating factors, such as the presence of corn grits as used in Diet IV. The concept that sugars do not initiate dental caries but serve mainly in the progression of caries is not new,

and has been reported previously.[5] However, the presence of corn grits alone does not seem to be the sole factor responsible for the greater experience of dental caries in the Group IV animals, for 5 per cent more corn grits are present in the animals receiving Diet II, yet there is significantly less dental caries. Muhler[5] has suggested that the most effective diet for inducing and extending dental caries in the rat is one containing both corn grits and sucrose, and the data obtained in this study seem to confirm these original observations.

The presence of 10 per cent yeast was associated with less dental caries in the males, but no reduction was found in the females when compared to animals receiving diets without yeast. However, since the experimental conditions were not similar to those used by previous workers,[1] no comparison between the studies is possible.

SUMMARY

Diets composed of ground barley and fish meal were studied for their effect on dental caries in the rat. It was shown that a diet composed of 50 per cent barley, 25 per cent fish meal, and 25 per cent sucrose was significantly less effective in initiating dental caries than a similar diet with only 5 per cent fish meal but to which 20 per cent corn grits had been added. The presence of sucrose, in the absence of initiating factors in the diet, did not increase the dental caries experience. The effect of adding 10 per cent yeast to the barley-fish meal diet was not associated with a significant reduction in dental caries.

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EFFECTS OF FLUORIDE AND NONFLUORIDE CONTAINING TIN SALTS ON THE DENTAL CARIES EXPERIENCE IN CHILDREN

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THROUGH a series of 6 separate clinical studies,[1-6] it has been shown that tin-containing fluoride compounds (stannous fluoride,[1, 3-5] stannous chlorofluoride,[2] potassium fluorostannite[6]) are effective agents for the partial reduction of dental caries in children when used topically in aqueous solution. Moreover, in 4 of these studies,[1-4] the tin-containing fluoride compound was superior to the anticariogenic effectiveness of sodium fluoride.

It is important to know why the stannous fluoride salts are superior to sodium fluoride for such knowledge may be useful in the search for more effective anticariogenic agents. The superiority of the tin fluoride compounds over sodium fluoride may be related in some manner to the cation. Experiments have demonstrated this concept in a variety of ways, such as studies with powdered enamel which show that stannous chloride has a marked antisolubility effectiveness while sodium chloride does not.[7] Similar solubility findings can be demonstrated by means of intact enamel surfaces,[8] or by changes in the permeability of the intact enamel surface following treatment with stannous chloride or sodium chloride.[9] Additional evidence could be presented to demonstrate the fact that the stannous ion itself, in the absence of the fluoride ion, has an inhibiting effect on acid solubility of enamel surfaces, is capable of producing a reduction in experimental rat dental caries, and can inhibit the acid solubility of intact rat molars. However, the real test of effectiveness of any compound rests in its ability to reduce human dental caries. Thus, the purpose of this paper is to describe the results of a clinical study comparing the effectiveness of stannous chlorofluoride and a nonfluoride stannous salt, a stannous sulfate complex salt with the formula $SnNaH(SO_4)_2Cl$ (hereafter referred to as stannous sulfate), in reducing the dental caries experience in young children.

EXPERIMENTAL

Approximately 750 children in Spencer, Indiana, between the ages of 6 and 15 years were divided into 3 groups having similar caries susceptibility.[10] Each child received a thorough dental prophylaxis and the first treatment solution was applied immediately thereafter. The next 3 treatments were completed within a 10-day period, but only the first was preceded by a prophylaxis.

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Group I received 4 applications of distilled water, Group II 4 applications of a 2 per cent solution of stannous sulfate, and Group III received 4 applications of a 4 per cent solution of stannous chlorofluoride. The pH of the various solutions received by the 3 groups were 6.8, 1.2, and 3.5, respectively. The pH of the solution of stannous chlorofluoride was not adjusted, but used as freshly prepared. The pH of the stannous sulfate solution was adjusted from its original pH of 1.8 to a pH of about 1.2 by the dropwise addition of a 25 per cent solution of sulfuric acid to a point where the solution was almost clear. The 2 stannous solutions were applied immediately after they were prepared, and if any solution remained after a 1-hour period it was discarded and fresh solution prepared. The applications were made to all the teeth in the mouth with a cotton swab, and the teeth were kept moist with the treatment solution throughout each 4-minute treatment period. In order to reduce variables between the 3 groups further, all of the children, as well as their families, used the same dentifrice throughout the entire experimental period. The dentifrice was a commercial nonfluoride-containing dentifrice packaged in plain cartons and tubes.

Each child received a complete 5 (or 7) film radiographic bite-wing examination at the beginning of the study, and at the 6-month and 12-month examination. All of the children were examined by the same dentist using the same criteria for dental caries diagnosis throughout the entire study. The dental caries were recorded on a record card with space available for 3 examinations all on the same form, so that the caries difference could be compared on a tooth-to-tooth basis as well as by a summarization process. During successive examinations, the examination form was masked so that the examiner and recorder had no knowledge of previous examination findings of treatment identity. The radiographic findings were added following the clinical examination and, following the initial examination, the mask was applied to the preceding examination so that each subsequent radiographic examination would be completely independent, as were the visual clinical examinations.

DATA AND DISCUSSION

The clinical observations are summarized in Table I and indicate the caries increments at the 6- and 12-month examination periods evaluated by the DMFT, DMFS, and the components of the DMFS indices, for the 3 treatment groups. These data indicate that only stannous chlorofluoride produced a significant reduction in the incidence of dental caries. Using the DMFT index, there was a 46.9 and 51.9 per cent reduction at the end of 6 months and 12 months, respectively, and a 32.5 and 36.5 per cent reduction at the same periods when the DMFS index was used. These reductions are all highly significant statistically as indicated by the low probabilities. Although the children receiving stannous sulfate appeared to have a tendency toward less dental caries, none of the reductions were significant.

It was somewhat unexpected to find that stannous sulfate was ineffective since in vitro studies previously performed suggested that it should have a

TABLE I

THE DENTAL CARIES INCREMENTS IN TERMS OF DMFT, DMFS, AND THE VARIOUS COMPONENTS OF THE DMFS INDEX IN CHILDREN RECEIVING EITHER A FLUORIDE CONTAINING TIN SALT OR A NONFLUORIDE CONTAINING TIN SALT AFTER 6 MONTHS AND 1 YEAR

GROUP	NO. OF SUBJECTS	DENTAL CARIES INCREMENT						PER CENT REDUCTION	
		DMFT	DMFS	PROXIMALS	OCCLUSALS	BU-LINGUALS	REVERSALS	DMFT	DMFS
				6-Month Data					
Water	231	0.96	1.91	0.86	0.69	0.35	0.27	—	—
Stannous sulfate	234	0.94	1.79	0.93	0.60	0.26	0.35	2.1 (0.50)*	6.3 (0.50)
Stannous chlorofluoride	220	0.51	1.29	0.55	0.42	0.32	0.49	46.9 (0.001)	32.5 (0.007)
				12-Month Data					
Water	181	1.81	3.70	1.86	1.15	0.67	0.16	—	—
Stannous sulfate	189	1.73	3.41	1.79	1.08	0.54	0.32	4.4 (0.50)	7.8 (0.44)
Stannous chlorofluoride	188	0.87	2.35	1.11	0.68	0.56	0.40	51.9 (0.00003)	36.5 (0.0009)

*Probability based upon the t test.

significant effect. As a result of the negative clinical studies, additional in vitro studies were performed in an attempt to learn more about the correlation between such tests and human clinical studies, since it is imperative to know if the findings of in vitro studies are reliable predictors of results from human clinical studies. Table II shows the results of some of these studies, and suggests that stannous sulfate has a pronounced antisolubility effectiveness provided that the concentration used is sufficiently high. When a 25 per

TABLE II

A SUMMARY OF ENAMEL SOLUBILITIES OF EMBEDDED RAT MOLAR TEETH AFTER TREATMENT IN VITRO WITH DISTILLED WATER, STANNOUS FLUORIDE, OR STANNOUS SULFATE*

COMPOUND	CONCENTRATION (%)	NUMBER OF HEMIJAWS	μG P DISSOLVED AFTER 20 MINUTES IN 10 ML. OF 0.2 N ACETIC ACID, pH 4.0	REDUCTION IN SOLUBILITY (%)
Water	—	11	70 ± 5†	—
Stannous fluoride	2	12	32 ± 1	54
Stannous fluoride	4	12	26 ± 2	63
Stannous fluoride	8	11	28 ± 1	60
Stannous fluoride	10	11	30 ± 2	57
Stannous sulfate	5	12	44 ± 4	37
Stannous sulfate	10	10	38 ± 2	46
Stannous sulfate	20	12	41 ± 3	42
Stannous sulfate	25	11	33 ± 5	53

*Method previously described.[11]
†Standard deviation.

cent solution of stannous sulfate was used, the antisolubility effectiveness was equal to stannous fluoride. Similarly, the effect of the topical application of stannous sulfate on reducing the acid solubility of intact rat molars in vivo and in vitro was compared to the effect of stannous fluoride, and showed approximately equal effectiveness (Table III). As a result of these studies, one would predict that aqueous solutions of stannous sulfate would produce a significant reduction in human dental caries when applied topically to the teeth. Furthermore, these latter studies suggest that perhaps one reason why stannous sulfate failed to produce a significant reduction in the present clinical study may be because of the use of such a low concentration of stannous ion (6,070 ppm Sn^{++}), furnished as stannous sulfate, when compared to such a high concentration of stannous ion (27,500 ppm Sn^{++}) as stannous chlorofluoride.

TABLE III

THE ACID SOLUBILITY OF INTACT RAT MOLARS FOLLOWING TOPICAL APPLICATION OF WATER, STANNOUS SULFATE, OR STANNOUS FLUORIDE*

COMPOUND	CONTRACTION (%)	MEAN μG P DISSOLVED		PERCENTAGE REDUCTION	
		IN VIVO	IN VITRO	IN VIVO	IN VITRO
Distilled water	—	71 ± 3.0†	37 ± 2.0	—	—
Stannous sulfate	2	41 ± 3.0	19 ± 1.4	42 (.001)‡	49 (.001)
Stannous fluoride	2	38 ± 1.0	15 ± 0.9	47 (.001)	60 (.001)

*Method previously described.[11]
†Standard deviation.
‡Probability based on t test.

CONCLUSIONS

The anticariogenic effectiveness of solutions of stannous sulfate (2 per cent) and stannous chlorofluoride (4 per cent) has been compared with distilled water when applied topically to children. The stannous chlorofluoride significantly reduced new caries development whereas the stannous sulfate was ineffective. The results of in vitro and in vivo animal studies using higher concentrations of stannous sulfate suggest that the failure of stannous sulfate to reduce dental caries in this study was a result of the low stannous ion concentration.

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THE AVAILABILITY OF BACTERIAL GROWTH FACTORS IN HUMAN GINGIVAL TISSUE

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IT has been pointed out that no increase in the activities of niacin, riboflavin, and pantothenic acid in saliva could be ascribed directly to residues of ingested food.[1] In a continuing study of the availability of bacterial nutrilites in the mouth, it was decided to investigate the role of the gingival mucosa in supplying such materials to its indigenous microflora.

MATERIALS AND METHODS

The organisms and media used were the same as described previously[1] except that sufficient agar to make a 2 per cent final concentration was added, thus making the assay system suitable for auxanograms. The inocula were grown on the surface of 125 ml. of solid assay media in Roux bottles. The cells were harvested by washing the agar surface, after 48 hours' incubation. A 10 ml. base layer composed of the basal medium without added vitamins was poured into sterile petri dishes. A seed layer containing 0.25 ml. of packed cells per 250 ml. of medium and all of the vitamins except the assay factor was overlaid in 4 ml. amounts The assay factor was pipetted in 0.1 ml. amounts to 1.2 cm. filter paper disks, placed strategically on the surface of the seed layer. Results were determined by measuring the diameter of the zone of growth which developed around each disk. Typical standard curves were obtained.

Samples of inflamed tissue were collected in the Department of Periodontics; samples of noninflamed tissue, in the Department of Surgery of the School of Dentistry. Immediately upon excision, each sample was divided into 2 portions. One was placed in formalin for eventual histopathologic study, the other wrapped in foil, identified, and frozen. Before assaying, each sample was weighed in a tared weighing bottle, then homogenized in 1 ml. of distilled water, using a Ten-Broeck tissue grinder. A small amount of powdered glass was used to facilitate grinding. The homogenates were decanted into heavy-walled Pyrex glass tubes and centrifuged at 2,500 r.p.m. for 10 minutes. Aliquots of the clear supernatant were pipetted to filter paper disks in 0.1 ml. volumes. Each specimen was assayed for its content of riboflavin, niacin, pantothenic acid, and biotin, as growth factor activity. Maximum response

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occurred after 24 hours' incubation of the assay plates. Results were calculated on the basis of 1 gram, wet weight, of tissue. A total of 33 samples were assayed, 18 of which were inflamed. In addition, several samples of each type were dried in a desiccator for 48 hours, then assayed in order to rule out any dilution effect of edema fluid.

During the examination of the donor, the existence of and depth of periodontal pockets was noted.

RESULTS

In view of the unavailability of information in the literature, it was decided that a gross comparison of the levels of activity in inflamed tissue samples with those of noninflamed tissue samples would provide the most satisfactory analysis of the data obtained. Some of the samples yielded negative assays for 1 or more growth factors. A number of these developed an apparent zone of inhibition in the faint undergrowth immediately adjacent to the filter paper disk. The per cent distribution of negative assays in noninflamed and in inflamed tissue samples is presented in Table I. It is notable that there is a marked increase in the occurrence of negative assays in noninflamed samples, when compared with inflamed samples.

TABLE I

THE PER CENT DISTRIBUTION OF NEGATIVE ASSAYS IN 15 NONINFLAMED AND 18 INFLAMED TISSUE SAMPLES

GROWTH FACTOR	PER CENT NEGATIVE	
	NONINFLAMED	INFLAMED
Riboflavin	66.6	11.1
Niacin	13.3	0
Pantothenic acid	26.6	5.5
Biotin	20.0	16.6

It was decided to delete all of the negative assays from further statistical consideration, since the occurrence of an apparent inhibitor in many of them rendered all of them suspect. The data listed in Table II demonstrates the differences in the growth factor activity measured in noninflamed tissue samples, as compared with inflamed tissue samples. The means and the extremes are presented.

The noninflamed tissue samples contained greater amounts of growth factor activity than did the inflamed tissue samples. An apparent anomaly exists in the case of pantothenic acid. However, the minimal values obtained for this factor, as shown in the range, were higher in noninflamed tissue samples than in inflamed tissue samples. Thus, there is conformity to the pattern of the other factors in this respect. Riboflavin appeared most significantly different, averaging 60 per cent less activity in inflamed tissue extracts as compared with noninflamed. Niacin activity was decreased about 33 per cent and biotin about 23 per cent. These differences cannot be explained as an edema fluid dilution effect, since the mean assay values of desiccated samples showed similar differences. In the latter instance, the pantothenic acid levels were decreased also. The negative values for riboflavin in the desiccated noninflamed tissues were related to the inhibiting factor mentioned earlier

TABLE II

THE AMOUNTS OF GROWTH FACTOR ACTIVITY (μg/Gm.) IN INFLAMED AND NONINFLAMED TISSUES

GROWTH FACTOR ACTIVITY	WET TISSUE		DESICCATED TISSUE	
	INFLAMED	NONINFLAMED	INFLAMED	NONINFLAMED
Riboflavin				
Mean	3.54	8.85	33.82	0*
Range	1.41-7.42	2.39-15.44		
No. samples	16	5	4	4
Niacin				
Mean	10.13	15.32	49.78	82.56
Range	2.42-21.88	7.93-26.97		
No. samples	18	13	4	4
Pantothenic acid				
Mean	14.05	13.48	10.87	13.96
Range	1.27-44.36	3.22-27.66		
No. samples	17	11	4	4
Biotin				
Mean	0.0242	0.0316	0.0869	0.1434
Range	0.0063-0.0625	0.0066-0.1292		
No. samples	15	12	4	4

*These results are related to the inhibiting factor mentioned in the text.

On the assumption that the depth of existing pockets is a measure of the severity of periodontal disease, hence inflammation, the inflamed tissue samples were divided into 2 groups, those excised from areas containing pockets of 5 mm. or less in depth, and those from areas containing pockets of 6 mm. or more in depth. The mean levels of activity of each group are compared with noninflamed tissue levels in Table III.

TABLE III

MEAN GROWTH FACTOR ACTIVITY (μg/Gm.) OF INFLAMED TISSUE IN RELATION TO PERIODONTAL POCKET DEPTH AS COMPARED TO THAT OF NONINFLAMED TISSUE

GROWTH FACTOR ACTIVITY	NON-INFLAMED TISSUE	INFLAMED TISSUE		PER CENT DIFFERENCE FROM NONINFLAMED	
		POCKET DEPTH 5 MM. OR LESS	POCKET DEPTH 6 MM. OR MORE	5 MM. OR LESS	6 MM. OR MORE
Riboflavin	8.85	2.90	4.59	−67.2	−48.1
Niacin	15.32	9.08	11.76	−40.7	−23.3
Pantothenic acid	13.48	18.49	7.72	+37.2	−42.7
Biotin	0.0316	0.0212	0.0276	−32.8	−12.7

These data suggest that greater amounts of growth factor activity occur as pocket depth increases. Here again, pantothenic acid activity appears anomalous, being greatly increased in samples from areas of relatively shallow pockets and greatly decreased in areas of relatively deep pockets.

DISCUSSION

The existence of a complex microflora, such as that of the mouth, must ultimately depend on a continuing supply of essential nutrilites. Cellular debris, derived from host tissue, probably furnishes appreciable amounts of such compounds. The occurrence of vitamins or vitamin-like compounds in gingival tissues, in bacterial growth factor forms, may reflect storage of these materials by the tissue cells. Thus, they would be available for cellular nutrition, and, by extension, for bacterial nutrition.

In general, noninflamed tissue samples assayed higher than inflamed tissue samples in their content of riboflavin, niacin, and biotin. The pantothenic acid content of both types of tissue was essentially the same. In addition, the noninflamed tissue samples assayed negatively with greater frequency than did the inflamed tissue samples. This was particularly noticeable in the case of riboflavin activity. The identification of an inhibiting substance, which was evidenced in many of the negative assay plates, may provide an explanation for the apparently paradoxical results. Since the organisms used as assay tools were oral streptococci, it is possible that the inhibitor will prove to be an immune body.

The differences in growth factor activity in inflamed gingival tissue, as compared with noninflamed gingival tissue, have no explanation thus far. The increased numbers of microorganisms on inflamed gingivae, described by Schultz-Haudt, Bruce, and Bibby,[5] among others, suggest the possibility that, under this circumstance, the organisms may compete more successfully with tissue cells for cell-contained nutrilites. Thus, the partial depletions noted might be brought about. Alternatively, the degradation and desquamation of tissue cells, for reasons other than infection, may be the depleting factor. A form of anachoresis, followed by bacterial proliferation, would then explain the increased populations described by Schultz-Haudt, Bruce, and Bibby.

The vitamin content of human tissue other than blood, measured microbiologically as growth factor activity, has had little prior attention. Only one report of this nature has been found in the literature. Taylor, Pollack, and Williams[2] were able to assay various fresh frozen tissues from healthy individuals who had died immediately following injury. Enzymatic hydrolysates were studied. Colonic mucosa, which was the tissue most nearly similar to gingival tissue assayed, produced levels of activity well within the ranges of the noninflamed samples reported herein.

Becks and Ratcliffe[3] reported a 20 per cent decrease in blood levels of pantothenic acid in periodontal patients. Similarly a reduction in niacin levels was evidenced, using retention methods. The decreased levels of niacin activity in inflamed gingival tissue reported here are in agreement with the results published. Further, it appears probable that other B vitamin concentrations are decreased also.

The results obtained with respect to pantothenic acid, however, do not coincide with the 20 per cent reduction reported.[3] Turesky, Crowley, and Glickman,[4] using histochemical methods, demonstrated an increase in –SH group content of proliferating epithelium in areas of gingival inflammation. In addition, there was noted a decrease in –SH groups in degenerating epithelium. The similarity between –SH group occurrence and the pantothenic acid levels described earlier (Table III) suggests the possibility of a change in the physiologic balance of pantotheine or coenzyme A in proliferating tissues. Pantotheine, which has a free –SH group, is the condensation product of pantothenic acid and mercapto-ethanolamine. The coupling of pantotheine to adenylic acid, through a pyrophosphate group, yields coenzyme A.

SUMMARY

An auxanographic method has been adapted for the microbiologic assay of growth factor activity in extracts of human gingival tissues. For purposes of evaluation, inflamed specimens were compared with noninflamed specimens with respect to activities measurable as niacin, riboflavin, biotin, and pantothenic acid.

The higher percentage of negative assays of noninflamed specimens is not yet understood.

In general, inflamed specimens assayed considerably lower than noninflamed specimens when the negative specimens were not considered in the analysis. The differences described could not be accounted for as a function of edema fluid dilution.

The pantothenic acid assay results appeared anomalous. Little difference occurred between the mean of the inflamed samples and that of the noninflamed samples. Those inflamed samples, taken from areas having pockets 5 mm. or less in depth, however, assayed higher, whereas those from areas having pockets 6 mm. or more in depth assayed lower than did the noninflamed samples.

The authors wish to express their appreciation of the cooperation received from Dr. R. L. Reeves and Dr. L. Hural who provided the necessary clinical services and advices.

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CALCIUM AND PHOSPHORUS METABOLISM IN THE *ia* RAT

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SINCE the first discovery of the *ia** mutant strain of rats by Greep,[1] Schour and his colleagues[2-6] have unfolded the major anatomic defects to be found in both the pre- and postnatal *ia* rat. These abnormalities, which are inherited by this strain as a simple mendelian recessive factor, appear to be due to a generalized defect in bone resorption. The failure of the incisor to erupt is not due to a defect in the tooth itself but due to a failure of the alveolar bone to undergo normal physiologic resorption. Bhaskar, Schour, Greep, and Weinmann[7] reported that administration of high doses of parathyroid extract at an early and opportune time had shown a corrective effect on the abnormalities in the *ia* rat. The purpose of the present study was to compare the calcium and phosphorus metabolism of the normal and *ia* rats in an attempt to elucidate the origin of the physiologic defect in the latter.

MATERIALS AND METHODS

The *ia* rats and normal littermate controls were obtained by breeding the homozygous males (genotype tt) with the heterozygous females (genotype Tt).† Out of 17 litters used in these studies, involving a total of 111 rats, the *ia* rats numbered 51 (27 males and 24 females) and the normal rats numbered 60 (37 males and 23 females). Unless otherwise stated all rats were maintained on a commercial laboratory chow, ground to facilitate consumption by the *ia* rats. The growth curves for the rats are given in Fig. 1. The curves for both male and female *ia* rats fall below those for the normal rats. Calcium was determined by an ethylenediamine tetraacetate method[8] and phosphorus by the method of Fiske and Subbarow.[9] Bones were prepared for analysis by removing both femurs from each rat and by carefully stripping them of adjacent soft tissue. The cleaned bones were wrapped in a piece of gauze, placed in a Soxhlet apparatus and extracted with an ether-ethanol (1:1) mixture for 24 hours followed by extraction with ether for 24 hours. The defatted bones were dried in an oven at 110° C. for several hours and weighed. Ashing of the bones was accomplished in a muffle furnace at 500 to 600° C. for 16 to 40 hours. After weighing the bone ash it was dissolved quantitatively in 100 ml. of dilute hydrochloric

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*Due to the apparent absence of the incisors these rats were first termed "incisorless animals," which was subsequently abbreviated to *ia*.

†T represents the dominant factor, incisors present; t the recessive factor, incisors absent.

acid. The ash solution was analyzed for calcium and for phosphorus after conversion of the latter to orthophosphate by treating the aliquots with 1 N hydrochloric acid at 100° C. for 10 to 12 minutes. Both femurs from each rat were pooled for the analyses. Blood was withdrawn by cardiac puncture and the serum used for analysis. Several animals were bled more than once with at least 4 days' interval between bleedings.

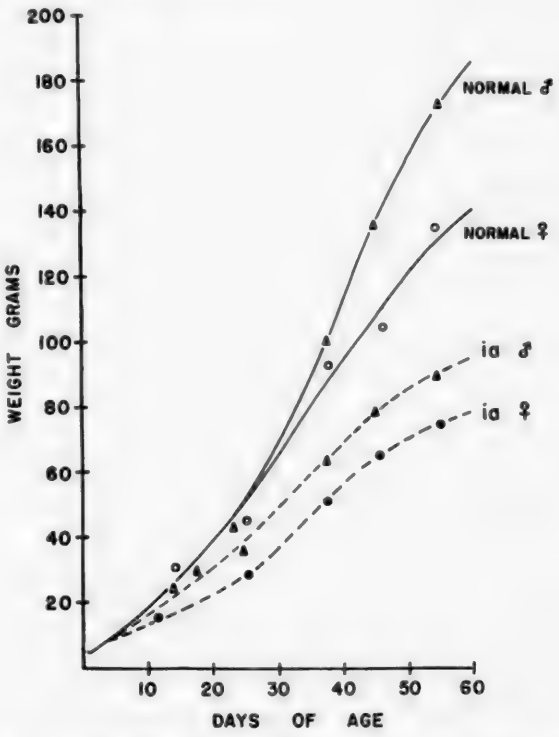

Fig. 1.—Growth curves for normal and *ia* male and female rats.

In the calcium depletion studies, the rats were placed on a low calcium diet (Table I) which was essentially the purified diet of Shaw[10] with the calcium salts omitted.

Parathyroidectomy was performed under ether anesthesia by hot wire cauterization.* The parathyroid extract (P 22) was prepared in these laboratories by hot acid extraction of bovine glands with 0.1 N HCl, 10 ml. per gram of fresh tissue, at 100° C. for 15 minutes extraction time, and contained 36 units per milliliter. It was administered subcutaneously in all cases. In the pair-feeding experiment, a single litter consisting of 4 *ia* and 4 normal rats was used. Each *ia* rat was paired with a normal rat of the same sex. The

*Details of the technic of parathyroidectomy will be supplied on request.

ia rat was permitted to eat ad libitum, its food consumption noted, and this quantity of food was fed to its littermate control the next day. Blood glucose was determined by a modification of the method of Nelson.[11]

At the time of sacrifice the heads from all the rats and the livers and pancreas glands from some of the rats were fixed in 10 per cent formaldehyde and kept for further studies.

TABLE I

LOW CALCIUM DIET

	% BY WEIGHT
Sucrose	68.4
Regular vitaminized rat casein (a)	24.0
Calcium deficient salt mixture (b)	2.6
Vitaminized corn oil (c)	5.0
a. *Regular vitaminized rat casein*	
To each 100 Gm. of regular rat casein add:	
Thiamine hydrochloride	1,458 μg
Riboflavin	1,458 μg
Pyridoxine hydrochloride	1,458 μg
Nicotinic acid	10.4 mg.
Calcium pantothenate	8.3 mg.
Choline chloride	417 mg.
Inositol	417 mg.
Para-aminobenzoic acid	125 mg.
b. *Calcium deficient salt mixture*	
Mix the following salts:	Gm.
K_2HPO_4	645
$NaH_2PO_4 \cdot H_2O$	126
$MgSO_4$	100
NaCl	282
Ferric citrate·H_2O	55
KI	1.6
$MnSO_4 \cdot H_2O$	0
$ZnCl_2$	1
$CuSO_4 \cdot 5H_2O$	0.6
c. *Vitaminized corn oil*	
To each 100 Gm. of corn oil add:	
β-carotene	22 mg.
Irradiated ergosterol	6,000 I.U.
2-methyl-1, 4-naphthoquinone	12 mg.
α-tocopherol	100 mg.

RESULTS

a. *Serum Calcium and Phosphorus Levels. Normal Diet.*—

Serum calcium and phosphorus values of *ia* rats and normal littermate controls, maintained on a normal diet, were determined at ages ranging from 10 to 53 days. For convenience of presentation the data have been arranged in age groups of 10-day intervals. The serum calcium levels (Fig. 2) were similar in both phenotypes from 10 to 39 and from 50 to 59 days of age. However, at 40 to 49 days of age, the serum calcium of the *ia* rat appeared to be significantly higher ($P < 0.02$) than the normal controls. The serum phosphorus levels (Fig. 3) showed a marked difference between the 2 phenotypes in the early age

groups. Between the ages of 10 and 39 days, the serum phosphorus levels of the *ia* rat were significantly lower ($P < 0.05$) than those of the normal controls.

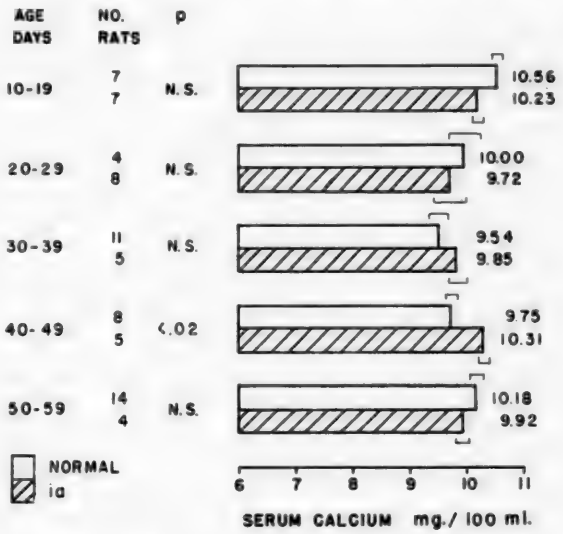

Fig. 2.—Serum calcium levels in the *ia* and normal rats at different ages. P represents the probability in Fisher's "t" test, NS indicating not significant ($P > 0.05$). The thin lines represent the standard errors of the mean.

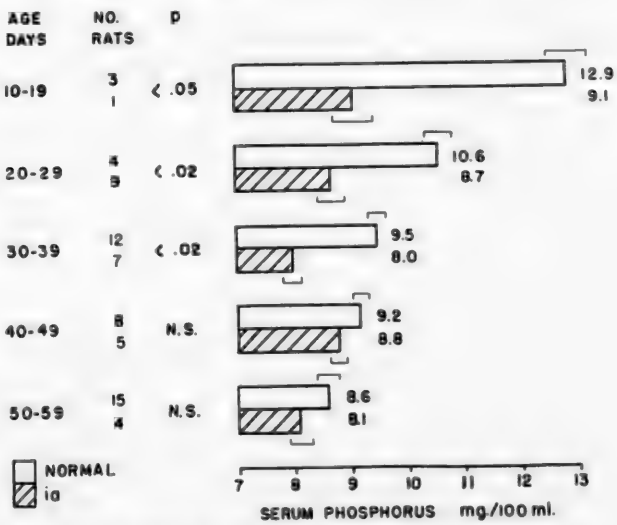

Fig. 3.—Serum phosphorus levels in the *ia* and normal rats at different ages. Legend as in Fig. 2.

Between 40 and 59 days this difference was not significant ($P > 0.2$). Whereas the serum phosphorus levels of the normal controls steadily decreased with increasing age those of the *ia* rats appeared to remain fairly constant.

b. *Bone Analyses. Normal Diet.*—

The bone ash contents, expressed as a percentage of the dry weight, of the femurs of rats ranging from 10 to 53 days of age were determined. Marked differences between the ash contents of *ia* rats and normal controls were revealed in the younger age groups (Fig. 4). The ash contents of the femurs from 20- to 49-day-old *ia* rats were significantly higher ($P < 0.05$) than those of normal controls. In the 10- to 19-day and 50- to 59-day age groups, there appeared to be no difference although the few rats in the latter group did not allow statistical comparison. Although the femurs from normal rats showed a steadily increasing ash content with increasing age, those from the *ia* rats appeared to reach a maximum value in the 30- to 39-day age group. There was a dramatic increase in ash content between the 20- to 29-day and the 30- to 39-day age group.

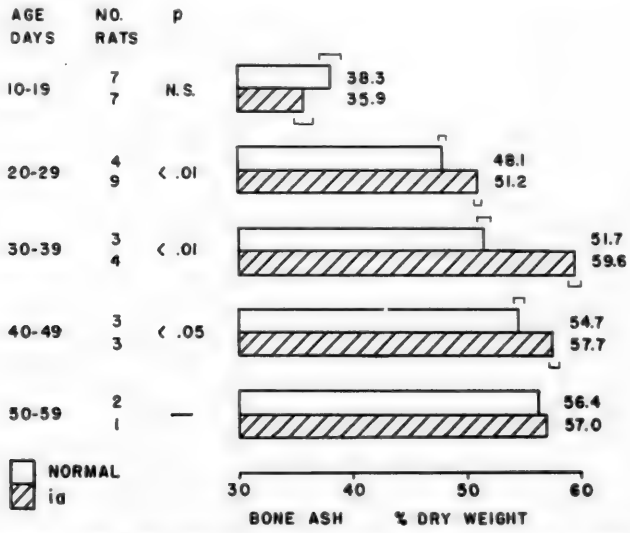

Fig. 4.—Bone ash contents, expressed as a percentage of the dry weight, of femurs from *ia* and normal rats. Legend as in Fig. 2.

The determination of the calcium and phosphorus contents of the femur ash did not uncover any marked differences between the phenotypes (Table II). Although the calcium content of the ash from the *ia* rats proved to be significantly higher ($P < 0.01$) than those from normal rats in the 10- to 19-day and 30- to 39-day age groups, it is felt that these differences are not important ones.

c. *Effect of Calcium Depletion, Parathyroidectomy, and Parathyroid Extract.*—

1. *Serum calcium and phosphorus:* Both *ia* and normal rats were subjected to calcium depletion followed by parathyroidectomy with and without the simultaneous administration of parathyroid extract. Calcium depletion was effected by placing the rats on a low calcium diet for either 4 or 13 to 14 days. Serum

TABLE II
CALCIUM AND PHOSPHORUS CONTENT OF BONE ASH

AGE (DAYS)	PHENOTYPE	NO. RATS	DAYS ON LCD	CALCIUM % ASH ± S. E.	P	PHOSPHORUS % ASH ± S. E.	P
Normal Diet							
10-19	Normal	7	0	33.2 ± 0.60	<.01	16.1 ± 0.41	NS
	ia	7		37.4		17.6	
20-29	Normal	3	0	35.4 ± 0.24	NS	18.4 ± 0.12	NS
	ia	8		35.9		18.1	
30-39	Normal	3	0	35.6 ± 0.16	<.01	18.2 ± 0.09	NS
	ia	4		37.0		17.9	
40-49	Normal	3	0	36.5 ± 0.09	NS	18.0 ± 0.11	NS
	ia	3		36.7		17.9	
50-59	Normal	2	0	36.2	--	18.1	--
	ia	1		36.6		18.5	
Low Calcium Diet (4 days)							
40-49	Normal	4	4	35.2 ± 0.31	NS	18.3 ± 0.11	<.05
	ia	4		35.6		17.7	
50-59	Normal	10	4	35.8 ± 0.23	NS	18.4 ± 0.24	NS
	ia	3		36.2		18.2	
Low Calcium Diet (13-14 days)							
37	Normal	2	14	32.7 ± 0.10	<.01	17.7 ± 0.20	NS
	ia	3		33.9		17.9	
48	Normal	6	13	34.9 ± 0.21	--	18.5 ± 0.18	<.05
	ia	4		34.9		17.5	
54	Normal	5	14	35.5 ± 0.28	NS	17.6 ± 0.25	NS
	ia	4		35.4		18.2	

LCD—low calcium diet; P—probability in Fisher's "t" test; S. E.—standard error of the mean; NS—not significant, $P > 0.05$.

calcium levels were determined in order to see if any differences, unobservable between the phenotypes in rats in a normal diet (Fig. 2), would be revealed under the stress of a low calcium intake. Calcium depletion for 4 days showed no differences between the phenotypes at 51 to 58 days of age. Depletion of calcium for 13 or 14 days, although resulting in no difference in the 48- to 54-day-old rats, did leave the serum calcium values of the *ia* rats higher in the 37-day-old rats (Fig. 5). Parathyroidectomy following 4 days on a low calcium diet reflected no difference between the serum calcium levels of the phenotypes 4 hours after the operation (Fig. 5). In parathyroidectomized rats which had been depleted of calcium by 4 or 13 to 14 days' administration of parathyroid extract at a dose of 25 units/100 Gm. body weight, no significant difference between the serum calcium levels of the 2 phenotypes was observed. No differences were observed between the serum phosphorus levels of the *ia* and normal rats following calcium depletion and parathyroidectomy with and without parathyroid extract administration (Fig. 6).

2. *Bone ash:* Calcium depletion reduced the ash contents of both *ia* and normal bones, 13 to 14 days' depletion having a much greater effect than 4 days' depletion (Fig. 7). The tremendous reduction in the bone ash of the

30- to 39-day-old *ia* rats as a result of 14 days' calcium depletion indicates that the *ia* rat bone, even though osteosclerotic at this age, is easily mobilizable by endogenous means.

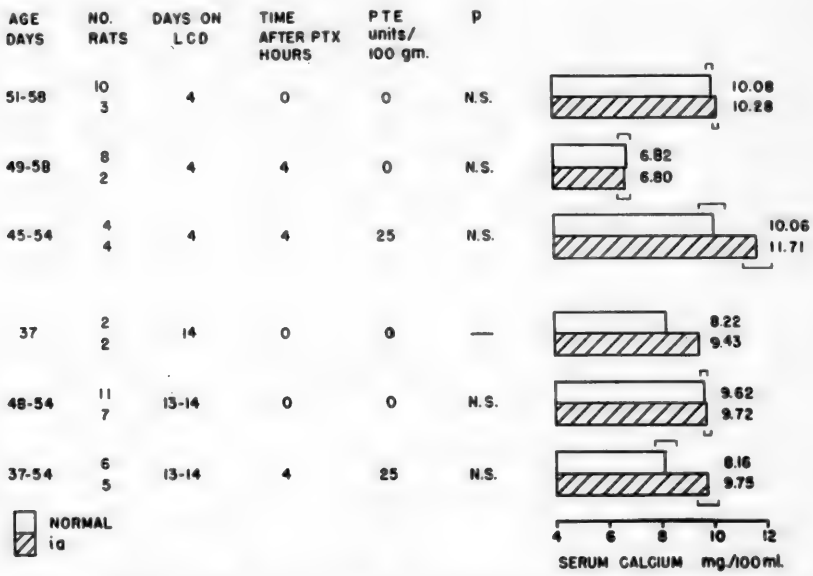

Fig. 5.—Serum calcium levels of *ia* and normal rats following calcium depletion and parathyroidectomy (PTX) with and without parathyroid extract (PTE) administration. LCD, low calcium diet. Remaining legend as in Fig. 2.

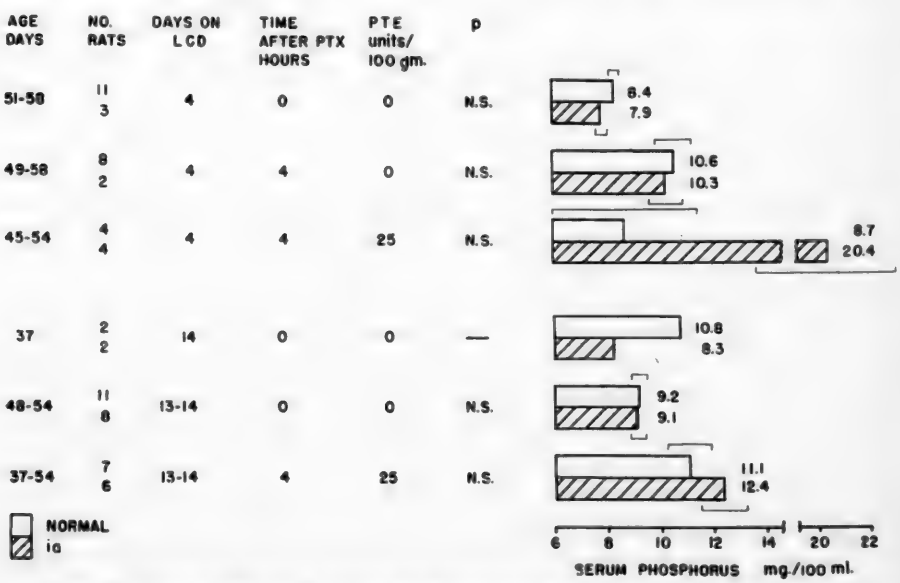

Fig. 6.—Serum phosphorus levels of *ia* and normal rats following calcium depletion and parathyroidectomy (PTX) with and without parathyroid extract (PTE) administration. Legend as in Fig. 5.

Calcium depletion did produce some significant differences between the calcium and phosphorus contents of *ia* and normal rat bone ashes (Table II), but, because of the indiscriminate pattern of these differences, no conclusions can be drawn from the data. The ratio of calcium to phosphorus content does not appear to be affected by calcium depletion.

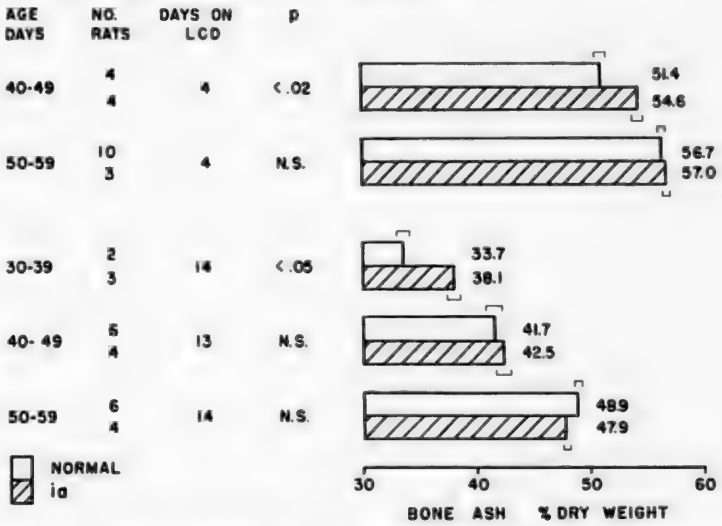

Fig. 7.—Bone ash contents, expressed as a percentage of the dry weight, of femurs from *ia* and normal rats following calcium depletion. Legend as in Fig. 2.

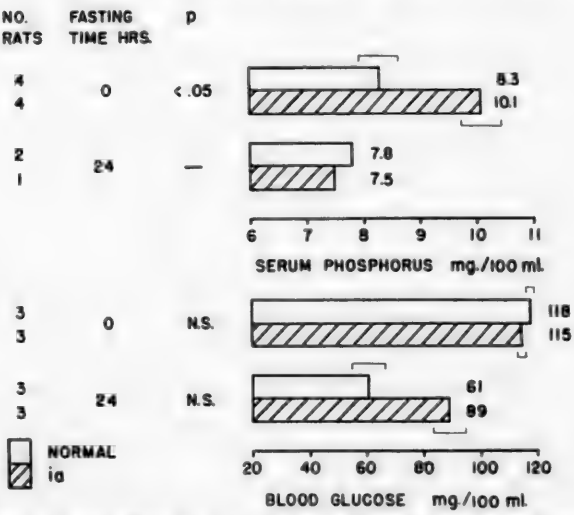

Fig. 8.—Effect of pair-feeding on the serum phosphorus and blood glucose levels in *ia* and normal rats. The rats, all from the same litter, were pair-fed for 9 days from 24 to 33 days of age. Remaining legend as in Fig. 2.

It must be noted that none of the rats used in this aspect of the study was under 37 days of age at the time of the determination of the serum levels. This fact modifies interpretation of the data.

d. *Serum Phosphorus and Blood Glucose After Pair Feeding.*—

A pair-feeding experiment was undertaken in an attempt to settle 2 points: (1) whether the difference in serum phosphorus between the phenotypes observed in the younger rats under normal conditions (Fig. 3) was of a fundamental pathophysiologic nature or merely a reflection of dissimilar dietary habits due to the handicap imposed on the *ia* rat by the absence of its incisors; and (2) whether a morphologic difference in the pancreas glands (slight vascular and cellular alterations in the isles of Langerhans) observed by Mohammed[12] would manifest itself in a difference in the blood glucose levels.

The rats, all from the same litter, were pair-fed a ground commercial chow for 9 days from 24 to 33 days of age. The *ia* rats gained an average of 11.8 Gm. of body weight while the normal controls gained an average of 4.2 Gm. during the 9-day period. The average daily food intake was 4.3 Gm. for the normal and 4.0 Gm. for the *ia* rats. Blood samples were taken for analysis before and after fasting for 24 hours. The serum phosphorus values of the fed *ia* rats were higher than those of the normal controls (Fig. 8), not lower as in animals which had been allowed food ad libitum (Fig. 3). No disparity between blood glucose levels in the fed or fasted rats was observed (Fig. 8).

DISCUSSION

This study of the calcium and phosphorus metabolism of the *ia* rat and its normal littermate control, although substantiating the earlier morphologic studies, did not unmask any major physiologic defect such as a parathyroid dysfunction which had been previously postulated.[7] The fact that the serum calcium levels in the *ia* rats were found not to be lower than those of the controls (in the 40- to 49-day-old group they were even higher) militates against the possible existence of a chronic hypoparathyroidism, particularly in view of the apparent lower food, and consequently calcium, intake of the *ia* rats. The lower serum phosphorus level in the *ia* rat at ages below 39 days (Fig. 3), although striking, was shown by a pair-feeding experiment (Fig. 8) to be largely dependent upon differences in dietary habits, at least at 24 to 33 days of age. However, the fact remains that the difference in serum phosphorus levels appears to exist in rats under 20 days of age. At this age it is difficult to visualize that the *ia* rat would be at any feeding disadvantage physically when the main source of nourishment could be expected to be the maternal milk. They may, of course, be at a disadvantage behaviorally.

However, even if a difference in dietary habits due to behavioral differences is granted it cannot be invoked as the only cause of the defect as morphologic evidence indicates the existence of the defect in utero[2, 3] where behavioral differences should be inoperative. In any case, a hypophosphatemia is indicative, if anything, more of hyperparathyroidism than hypoparathyroidism. The fact that the serum phosphorus level of the *ia* rat in the pair-fed animals (10.1 mg. per cent) was higher than that in the same age group which had not been pair-fed (8.0 mg. per cent) is difficult to explain except on a behavioral basis. In the pair-fed experiments, the *ia* rats were individually caged in contrast to all other occasions when the rats were maintained as complete litters in a cage; perhaps the former conditions were conducive to a better appetite.

During the 40- to 49-day-age period, 3 events happened which may or may not be causally linked: (1) the serum phosphorus levels cease to be significantly different (Fig. 3); (2) the bone ash content of the *ia* rat after reaching a maximum at 30 to 39 days of age is in decline (Fig. 4); and (3) the serum calcium levels are significantly higher in the *ia* rat for the first and only time. All these events are consistent with a sudden increase in net bone resorption due to increased parathyroid activity. Previous to this there was a precipitous rise in the bone ash content of the *ia* femur (30 to 60 per cent) during the period from 10 to 39 days of age, compared with a more gradual rise in that of the normal femur (38 to 52 per cent). These findings are in conformity with the morphologic studies in the tibia of the *ia* rat in which it was found that there was an increase in the extent of osteosclerosis up to the age of 30 days, followed by a steady decrease after 30 days of age.[4]

The studies of the effect of calcium depletion, parathyroidectomy, and parathyroid extract are limited in their interpretable value due to the ages of the rats involved. The youngest rats were 37 days of age and, although it can be concluded that at this age the serum calcium levels did not reflect the existence of hypoparathyroidism (Fig. 5), it is quite possible that the physiologic defect sought for might have disappeared by this time. However, since some of these rats had been placed on a low calcium diet at the age of 23 days, it is unlikely that they would have survived had a chronic hypoparathyroidism existed at this age. Calcium depletion naturally could not be instituted much before 23 days due to the suckling period of 21 days. The fact that the serum calcium levels of the *ia* rats fall to a similar extent to those of normal rats suggests normal functioning of the parathyroid glands at this age (49 to 58 days), for had the parathyroids been hypofunctioning, parathyroidectomy should have had little effect on the serum calcium levels in the *ia* rat. Calcium depletion followed by parathyroidectomy with parathyroid extract administration resulted in serum calcium levels which were not significantly different. This would suggest that the bones of the *ia* rat show no lack of response to resorption by parathyroid hormone, at least to hormone of bovine origin.

In a study of rib transplants between normal and grey-lethal mice (which exhibit a genetic resorption deficiency similar to the *ia* rat), Barnicot[13] concluded that the defect lay in the hormonal environment rather than in the bone tissue itself, since grey-lethal ribs transplanted to the normal host showed resorption. However, in a similar study employing the *ia* rat, Gillette, Mardfin, and Schour[14] pointed out that the manifestation of resorption on the part of an *ia* transplant in a normal animal does not settle the question as to whether the defect is in the tissue or in the hormonal environment, since normal host cells may invade the transplant as well as host hormone. They detected some resorption behavior on the part of normal transplants in *ia* hosts and conclude that the behavior of the transplants in various situations could best be accounted for by postulating a deficiency in the *ia* osteoclasts rather than in the *ia* parathyroid hormone levels. They suggested that *ia* osteoclasts are sluggish at normal hormone levels but respond to the excessive hormone levels such as those that would be produced by the massive parathyroid extract injections

of Bhaskar, Schour, Greep, and Weinmann.[7] The *ia* condition has been described as exhibiting reduced osteoclasis rather than total absence of osteoclasis. Bhaskar, Mohammed, and Weinmann[15] in comparing the staining properties of normal and of *ia* osteoclasts found that the cytoplasm of *ia* osteoclasts showed less uptake of thionin, which may be indicative of a lower RNA content. This finding in itself, however, would not indicate whether this difference is intrinsic to the cell itself or induced by a difference in hormone level.

The interpretations may be summarized as follows: Studies of the calcium and phosphorus metabolism of the *ia* rat did not reveal the nature of the physiologic defect responsible for the generalized lack of bone resorption. The defect did not appear to reside primarily in either the existence of a chronic hypoparathyroidism or a resistance of the bone to resorption by parathyroid hormone. On the contrary, the older *ia* rats appeared to have available as great a store of mobilizable calcium as suggested by observations following calcium depletion and parathyroid extract administration. Using bone ash content as the criterion, the defect appeared to be present at least until 39 days of age but, of course, it is possible that abnormal bone ash contents would lag behind the removal of the defect and thus not be an accurate reflection of the existence of the defect. By all the criteria investigated, with the exception of body weight, the *ia* and normal rats tended to become similar by the age of 60 days. It appears that further investigations might well focus on the nature of the organic matrix of the *ia* bone as well as calcium balance and calcium turnover studies.

SUMMARY

1. The calcium and phosphorus metabolism of *ia* and normal rats were studied by determining the serum calcium and phosphorus levels in rats which had been maintained on normal diet and in those which had been calcium depleted and parathyroidectomized with and without the simultaneous administration of parathyroid extract.

2. No differences between the serum calcium levels of the *ia* and normal rats maintained on normal commercial chow were observed from 10 to 59 days of age with the exception of the 40- to 49-day age group in which those of the *ia* rat were higher.

3. The serum phosphorus levels of the *ia* rats were found to be consistently lower than those of the normal rats below the age of 40 days. This difference was shown by a pair-feeding experiment, in which the direction of the difference was reversed, to be largely dependent upon feeding habits.

4. Bone analyses revealed the *ia* rat femur to be hypercalcified relative to the normal rat femur up to the age of 49 days. A rapid rise in the bone ash content of the *ia* rat femur from 10 days of age to a maximum in the 30- to 39-day age period was followed by a steady decline to normal values by the 50- to 59-day age period. Such findings indicated a sudden increase in net resorption of the *ia* bone following the 30- to 39-day age period and the possible disappearance of the fundamental defect by this time.

5. Determination of the calcium and phosphorus contents of the bone ash suggested no significant differences between the 2 phenotypes with the exception of the bone ash calcium contents in the 10- to 19-day and 30- to 39-day age groups in which those of the *ia* rats were higher than normal.

6. Calcium depletion for either 4 or 13 to 14 days uncovered no strain on the serum calcium levels of the *ia* rat relative to that of the normal rat.

7. The administration of bovine parathyroid extract following calcium depletion and parathyroidectomy gave serum calcium levels in the *ia* rat as high as, if not higher than, those in the normal rat. This observation suggested that the *ia* rat bone responds to resorption by parathyroid hormone.

8. It is concluded that the generalized defect in bone resorption in the *ia* rat is not due primarily either to the existence of a chronic hypoparathyroidism or to a resistance of the *ia* rat bone to resorption by parathyroid hormone.

The authors are indebted to Miss Anna Morse and Dr. Roy O. Greep for supplying the initial rats for our breeding colony, to Dr. Ernest E. Adams for the details of the bone defatting and drying procedure, to Eli Lilly and Company for frozen beef glands, and to Dr. Ernest Knobil and Miss Mary Hagney for blood glucose determinations.

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THE CLINICAL DENTAL TRIAL

II. Evaluation of a Relaxing Drug for Operative Dentistry

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THE evaluation of the effects of tranquilizing drugs is subject to tremendous variation both in the effect on the patient and in the observation of the clinician. As a result, some observers feel that the effectiveness of ataractic drugs is either extremely limited or nonexistent, while others believe they are the answer to the problems of the management of the apprehensive patient.

It is the purpose of this paper to describe a study of the clinical effectiveness of a new drug in making patients more relaxed for operative dentistry. Particular emphasis will be placed upon the design of the experiment.

DESCRIPTION OF THE EXPERIMENT

Many patients will be fairly relaxed in the dental chair, particularly after local anesthesia has been administered and the dentist has the confidence of the patient, yet, in spite of a carefully cultivated approach by an experienced dentist and absence of pain in the tooth or teeth to be worked upon, some patients remain very apprehensive and tense. The reason for this behavior may be basic to each patient, or due to previous adverse dental experience.

A new drug, which had been shown to be relatively free from noxious side effects and "hang-over" and at the same time to possibly act as a relaxant in medical practice, was offered as a means to overcome tenseness and noncooperation in the dental chair. Members of the Division of Operative Dentistry of the School of Dental and Oral Surgery (under the leadership of H. Sherman)[1] performed an objective study of the effectiveness of this agent in the school clinic and, to some extent, in their private offices.

In order to limit the study to those patients who were in need of such medication, a roster of such patients was drawn by listing all tense and difficult patients who were admitted to the clinic during a period of several months. A total of 40 patients, age 11 to 65, 13 male and 27 female, was used. By means of a prearranged randomized scheme, each patient received, about 20 minutes before his next dental visit was begun, a sealed, coded envelope containing a capsule. The envelope was attached to a card to be filled in by a member of the dental evaluating team.

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Several hundred cards together with their attached envelopes were numbered consecutively. Half of these were to be assigned to the placebo capsules and the other half to the drug capsules. The specific numbers actually assigned were picked from a table of random numbers so that there would be an equalization after every 10 cards. Thus, for the first 20 cards, the numbers 1, 2, 3, 9, 10, 11, 13, 14, 19, and 20 were assigned to placebo capsules and numbers 4, 5, 6, 7, 8, 12, 15, 16, 17, and 18 were assigned to the drug capsules. The envelope, enclosure, and card were dispensed in numerical sequence by the dental nurse.

Twenty-five patients returned for more than 1 treatment visit during the study (Table I). Each time, they received an appropriately numbered envelope and enclosed capsule, without regard to what they had received before. It would have been preferable to dispense a capsule other than what had been given the previous time, but such a procedure would have been much more difficult administratively and would have lent itself more readily to the breaking of the code and the negation of the objectivity of the test.

TABLE I

NUMBER OF TREATMENT VISITS

NUMBER OF TREATMENT VISITS	NUMBER OF PATIENTS
1	15
2	14
3	4
4	2
5	2
6	1
9	1
14	1
Total	40

No one connected with the clinical study knew whether the capsule contained the relaxing drug being studied or a placebo. Even during the final evaluation of the results, the code was broken only to distinguish between capsule A and capsule B, without defining which contained the "active" agent.

In addition to the usual information (i.e., name, sex, age, type of work performed) the degree of relaxation was noted by one of the dental evaluators who encircled either ++, +, or –, from sufficient relaxation to no relaxation. Prior to the initiation of the study, the dental evaluators attempted to standardize their interpretation of these notations.

ANALYSIS OF THE RESULTS

In analyzing the results of a study of this type, one very difficult problem always arises: How is relaxation to be defined? While this poses great difficulties, it is much simpler to obtain information as to comparative relaxation. Thus, ++ denotes more relaxation than +, which in turn denotes more

than –. Another problem lies with the large number of revisits. There was a total of 102 treatment visits, of which more than half (59) occurred with 11 patients.

One way to solve both problems would be to utilize only those patients with revisits who received a different drug on the second visit than they had the first time. There were 20 such patients. Thus, we have 20 pairs for comparison in which the pairing is in the same individuals based upon 2 different treatments (drug and placebo). Because of the randomized manner in which the drug and placebo were administered, there was no systematic order in which each patient received the capsule (i.e., drug the first time and placebo the second time). By this technic of pairing, the response of each patient with one treatment was compared with his response to the other treatment, in terms of the notation of the evaluator on each card. Thus, ++ on one card denoted more relaxation than + on the other card, + denoted more than –. Whenever the same notation was found on each pair member's card, this was considered a tie.

Of the 20 pairs, 9 favored the drug, 4 favored the placebo, and 7 were tied. The significance of the results can be tested by the normal curve[2] by comparing the proportion of untied pairs favoring the drug with one half, the proportion that ought to favor the drug if there were no difference between the degree of relaxation with the drug and with the placebo.[3] Since the variable under scrutiny is a discrete one and the number of untied pairs is small, a continuity correction[4] may be applied, reducing the 9 untied pairs by 0.5. The relative deviate, Dev/σ, is calculated as

$$\text{Dev}/\sigma = \frac{(9 - 0.5) - np'}{\sqrt{np'q'}}$$

where $n = 9 + 4 = 13$ untied pairs

and $p' = q' = 0.5$, so that

$$\text{Dev}/\sigma = \frac{8.5 - (13)(0.5)}{\sqrt{(13)(0.5)(0.5)}} = \frac{2}{1.8} = 1.1.$$

Since the ratio Dev/σ is less than 2.58, the difference between the number of untied pairs favoring the drug and those favoring the placebo in terms of greater relaxation is statistically not significant.

A convenient alternative to the normal curve test in this case is to calculate χ^2 (with one degree of freedom) as

$$\chi^2 = \frac{[(9 - 4) - 1]^2}{9 + 4} = \frac{16}{13} = 1.2.$$

With 1 degree of freedom, $\text{Dev}/\sigma \sqrt{\chi^2}$.

Another way of avoiding the necessity of defining relaxation and of avoiding the complication of the excessive number of revisits is to ignore the revisits completely. The patients may be paired artificially in order of the sequence

of their presenting for treatment at the first treatment visit (i.e., the first patient receiving the new drug with the first receiving the placebo, etc.).[5] Pairing in this manner, 19 pairs of patients were obtained, with 5 pairs showing greater relaxation with the drug, 6 pairs greater relaxation with the placebo, and 8 pairs tied with respect to degree of relaxation. It should be noted that this group of patients includes some utilized under the previous pairing scheme. Calculating χ^2 as in the previous pairing:

$$\chi^2 = \frac{[(6 - 5) - 1]^2}{6 + 5} = 0.0.$$

Thus, this type of pairing also shows no statistically significant difference between the degree of relaxation obtained with the drug or the placebo.

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THE EFFECT OF A MODIFIED STANNOUS FLUORIDE-CALCIUM PYROPHOSPHATE DENTIFRICE ON DENTAL CARIES IN CHILDREN

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PREVIOUS studies have demonstrated that a stannous fluoride-calcium pyrophosphate dentifrice will produce a significant reduction in dental caries in children.[1-3] A similar dentifrice described by Jordan and Peterson also produced a significant reduction in dental caries.[4] In contrast, a stannous fluoride-dicalcium phosphate dentifrice,[5] or a sodium fluoride dentifrice with either calcium carbonate,[6, 7] dicalcium phosphate,[8] or calcium pyrophosphate[3] as the abrasive did not significantly reduce dental caries. These studies suggest that the abrasive as well as the fluoride is important in preparing a dentifrice which can reduce dental caries.

In considering the potential chemical reactions within a fluoride dentifrice, it was thought that the addition of a small amount of a calcium sequestering agent, such as sodium metaphosphate, might help maintain the fluoride ion in an active form. Thus, the purpose of this study was to determine the clinical effectiveness of a stannous fluoride-calcium pyrophosphate dentifrice to which a small amount of insoluble sodium metaphosphate had been added.

EXPERIMENTAL

Approximately 600 children, ages 6 to 15 years, who resided in Bloomington, Indiana, were divided into 2 groups according to previous dental caries susceptibility as previously described.[1] The clinical procedures, the type of examination and examination form, and the precautions used to eliminate bias both from the examination and through the distribution of the two dentifrices have been previously described.[1-3, 5, 9] All of the children were reexamined at 6 and 12 months. The composition of the 2 dentifrices used throughout this study are seen in Table I. The age and sex distribution of the children in the 2 groups at the initial examination is seen in Table II.

DATA AND DISCUSSION

The data obtained in this study are found in Table III. These data indicate a significant reduction in the incidence of dental caries both at the end of 6 months and at the end of a 1-year period through the use of the stannous fluoride dentifrice which contains a small amount of insoluble sodium

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TABLE 1

COMPOSITION OF THE EXPERIMENTAL DENTIFRICE

DENTIFRICE CONSTITUENT	CONTROL (PER CENT)	EXPERIMENTAL (PER CENT)
Calcium pyrophosphate	40.0	38.0
Stannous pyrophosphate	0	1.0
Insoluble metaphosphate	0	1.0
Detergent (nonsoap)	1.5	1.5
Humectant	30.0	30.0
Binder	1.4	1.4
Water	26.0	26.0
Stannous fluoride	–	0.4
Flavor	q.s.	q.s.

TABLE II

AGE DISTRIBUTION OF SUBJECTS AT THE BEGINNING OF THE STUDY, AFTER 6 MONTHS, AND AFTER 12 MONTHS

AGE AT START OF TEST	NO. OF SUBJECTS AVAILABLE AT START		NO. OF SUBJECTS AVAILABLE AFTER 6 MONTHS		NO. OF SUBJECTS AVAILABLE AFTER 12 MONTHS	
	CONTROL	SnF_2	CONTROL	SnF_2	CONTROL	SnF_2
5	1	0	1	0	1	0
6	25	28	23	24	19	20
7	46	41	44	39	37	34
8	37	43	33	36	28	33
9	23	34	23	34	23	31
10	31	21	28	16	24	18
11	35	34	29	31	26	29
12	27	32	24	29	21	26
13	23	21	20	18	16	16
14	19	21	14	22	12	20
15	16	17	13	16	13	16
16	11	11	11	10	10	9
17	2	3	2	3	2	3
18	0	3	0	3	0	2
All ages	296	309	265	281	232	257

TABLE III

THE EFFECT OF A STANNOUS FLUORIDE-CALCIUM PYROPHOSPHATE DENTIFRICE CONTAINING 1 PER CENT INSOLUBLE METAPHOSPHATE ON THE DENTAL CARIES EXPERIENCE OF CHILDREN

GROUP	NO. OF SUBJECTS	DENTAL CARIES INCREMENTS								
		DMFT	REDUCTION (PER CENT)	P	DMFS	REDUCTION (PER CENT)	P	PROXIMAL	OCCLUSAL	BUCCAL-LINGUAL
After 6 Months										
Control	262	1.07	28	0.01	2.33	22	0.03	1.19	0.72	0.41
SnF_2	282	0.77			1.83			0.96	0.62	0.25
After 12 Months										
Control	231	2.12	25	0.01	4.90	23	0.007	2.70	1.46	0.74
SnF_2	256	1.60			3.79			2.05	1.20	0.54

metaphosphate. When the DMFT index is used for evaluating the caries experience in the 2 groups at the end of 1 year, a reduction of 25 per cent is obtained, and when the DMFS index is used, the reduction is 23 per cent. Similarly, each of the components of the DMFS index shows reductions in the dental caries experience.

Since the dentifrices tested previously by the author and by Jordan and Peterson were not tested simultaneously and, of course, were tested independently from this study, a comparison of the clinical results is not possible. In all cases the reduction in dental caries was statistically significant when a stannous fluoride-calcium pyrophosphate dentifrice was used. From the result of this study it does not appear that the addition of a small amount of a calcium sequestering agent improved the clinical effectiveness of the dentifrice.

SUMMARY

A stannous fluoride-calcium pyrophosphate dentifrice containing in addition 1 per cent of a calcium sequestering agent, insoluble sodium metaphosphate, has been clinically tested. When used by 6- to 15-year-old children for 1 year the reduction was 25 and 23 per cent, respectively, when the DMFT and DMFS indices were used. Both reductions were statistically significant.

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THE RELATIONSHIP OF THYROID ACTIVITY TO THE DENTAL CARIES EXPERIENCE IN MICE

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PREVIOUS studies have clearly indicated that the activity of the thyroid gland is related to the dental caries experience in the rat.[1-3] In order to extend these findings to other rodents it was decided to repeat some of the original studies in the mouse, since dental caries studies in the hamsters are so difficult to repeat with reproducible accuracy. While the mouse is seldom used in experimental dental caries studies due to the small size of the molars, caries in this animal have been reported.[4]

EXPERIMENTAL

A total of 125 weanling male Swiss albino mice were divided into 5 experimental groups according to body weight. The 5 groups of mice received experimental diets (Table I) for 100 days, after which time they were sacrificed by ether and their heads removed for dental caries evaluation by methods previously described for the rat.[5] All of the mice were housed in pairs in raised screen cages in an air-conditioned room.

TABLE I

COMPOSITION OF THE EXPERIMENTAL DIETS

DIETARY COMPONENTS	EXPERIMENTAL GROUPS				
	I	II	III	IV	V
	(Gm./6 Kg.)				
Whole milk powder	1,710	1,800	1,800	1,800	1,800
Yellow corn (I. U. ground)			3,840	3,836	3,840
Yellow corn (grits)	2,760				
Corn meal		3,840			
Granulated sugar	1,170				
Alfalfa	288	288	288	288	288
Irradiated yeast	12	12	12	12	12
Sodium chloride, iodized	60	60	60	60	60
Desiccated thyroid					*
Propylthiouracil				6	

*Fed in increasing amounts from 10 to 20 mg. per day depending upon growth of the animal.

RESULTS AND DISCUSSION

The results of this study are seen in Table II. In no group was there a significant number of carious lesions. These results are markedly different from those obtained using the rat in which it was shown that thiouracil significantly increases dental caries. The reason for these differences are of

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Since the dentifrices tested previously by the author and by Jordan and Peterson were not tested simultaneously and, of course, were tested independently from this study, a comparison of the clinical results is not possible. In all cases the reduction in dental caries was statistically significant when a stannous fluoride-calcium pyrophosphate dentifrice was used. From the result of this study it does not appear that the addition of a small amount of a calcium sequestering agent improved the clinical effectiveness of the dentifrice.

SUMMARY

A stannous fluoride-calcium pyrophosphate dentifrice containing in addition 1 per cent of a calcium sequestering agent, insoluble sodium metaphosphate, has been clinically tested. When used by 6- to 15-year-old children for 1 year the reduction was 25 and 23 per cent, respectively, when the DMFT and DMFS indices were used. Both reductions were statistically significant.

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THE RELATIONSHIP OF THYROID ACTIVITY TO THE DENTAL CARIES EXPERIENCE IN MICE

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PREVIOUS studies have clearly indicated that the activity of the thyroid gland is related to the dental caries experience in the rat.[1-3] In order to extend these findings to other rodents it was decided to repeat some of the original studies in the mouse, since dental caries studies in the hamsters are so difficult to repeat with reproducible accuracy. While the mouse is seldom used in experimental dental caries studies due to the small size of the molars, caries in this animal have been reported.[4]

EXPERIMENTAL

A total of 125 weanling male Swiss albino mice were divided into 5 experimental groups according to body weight. The 5 groups of mice received experimental diets (Table I) for 100 days, after which time they were sacrificed by ether and their heads removed for dental caries evaluation by methods previously described for the rat.[5] All of the mice were housed in pairs in raised screen cages in an air-conditioned room.

TABLE I

COMPOSITION OF THE EXPERIMENTAL DIETS

DIETARY COMPONENTS	EXPERIMENTAL GROUPS				
	I	II	III	IV	V
			(Gm./6 Kg.)		
Whole milk powder	1,710	1,800	1,800	1,800	1,800
Yellow corn (I. U. ground)			3,840	3,836	3,840
Yellow corn (grits)	2,760				
Corn meal		3,840			
Granulated sugar	1,170				
Alfalfa	288	288	288	288	288
Irradiated yeast	12	12	12	12	12
Sodium chloride, iodized	60	60	60	60	60
Desiccated thyroid					*
Propylthiouracil				6	

*Fed in increasing amounts from 10 to 20 mg. per day depending upon growth of the animal.

RESULTS AND DISCUSSION

The results of this study are seen in Table II. In no group was there a significant number of carious lesions. These results are markedly different from those obtained using the rat in which it was shown that thiouracil significantly increases dental caries. The reason for these differences are of

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real interest especially since the salivary glands of the mouse are structurally different from those in the rat and since the salivary glands have been suggested as intermediants in the thyroid-dental caries relationships.

TABLE II

THE EFFECT OF FEEDING DIFFERENT EXPERIMENTAL DIETS ON THE DENTAL CARIES EXPERIENCE IN MICE

EXPERIMENTAL GROUP	NO. OF MICE	WEIGHT GAIN (GM.)	MEAN NO. DENTAL LESIONS
I	11	29	0.0
II	16	30	0.1
III	19	28	0.0
IV	14	33	0.0
V	14	23	0.2

The mice receiving sucrose (Group I), or those receiving a corn meal diet (Group II), or ground corn (Group III), also had no caries. Apparently the initiation of dental caries in the mouse is significantly different from that in the rat. Differences in particle size of the corn does not seem to explain the differences.

CONCLUSIONS

The administration of thiouracil to weanling mice, in the same amounts used in experiments showing an increase in caries in rats, did not induce dental caries. The addition of sucrose, fine corn meal or ground corn which contains particles of varying sizes, also did not result in dental caries in the mouse.

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THE HYALURONIDASE ACTIVITY OF SALIVA

VI. Effect of Antibiotic Therapy on Hyaluronidase Activity of Saliva

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THE hyaluronidase activity of saliva has been evaluated for young adults and found to be relatively constant as well as reproducible over a period of weeks within a given range of activity for the individual.[1] A direct relationship of hyaluronidase activity of saliva has been demonstrated to the gingivitis of the subject and to the oral hygiene of the subject.[2]

A sharp increase in the incidence of streptococcal upper respiratory infections occurred in recruits in each of the 4 regiments of the Center in January, 1955, and a mass antibiotic prophylaxis program was undertaken. This took place coincidentally with salivary hyaluronidase studies on selected groups of recruits; therefore an opportunity to study the effect of antibiotic therapy upon this enzyme was afforded.

It is the purpose of this study, therefore, to determine the effect of penicillin therapy upon the hyaluronidase activity of saliva of young adults.

METHOD

The subjects for this study were naval recruits undergoing training at the U. S. Naval Training Center, Bainbridge, Maryland, from January, 1955, to May, 1955. Two companies of naval recruits with an average age of 18 years were selected for study, one company which received 1 intramuscular dose of a long-acting penicillin and a second company which received oral penicillin continuously for 31 days.

Saliva was collected for each test by paraffin stimulation at 11 o'clock in the morning after 5 hours of supervised fasting. This report presents the results of the different tests summarized separately by collection and by company.

The first company of 56 young male adults had saliva collected on 3 successive weeks All members of the group received 1 injection of a long-acting penicillin (benzathine penicillin G in aqueous suspension*) 3 days prior to the second saliva collection. The dosage for recruits with even service numbers was 600,000 units and 900,000 units for those with odd service numbers. No other drugs were utilized during this period. The second and third samples of saliva, thus, were taken from recruits having received penicillin 3 days prior

The opinions or assertions contained in this article are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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*Bicillin, Wyeth Laboratories, Inc.

and 10 days prior to the collections, respectively. At the time of the beginning of these tests, the incidence of Group A streptococcus, as determined by throat culture surveys of recruits in the regiment from which this group was selected, was 13 per cent.[3]

The second company of 49 young male adults had saliva collected on 3 separate occasions, the first at a 14-day interval and the second at a 31-day interval from the time of the initial collection. All members of the group were placed on oral penicillin between the first and second test. Oral penicillin (crystalline penicillin G potassium buffered with calcium carbonate*) was administered to the group in dosages of 500,000 units per day beginning 4 days after the first test.[3] This was given in divided dosages, 250,000 units upon arising and 250,000 units upon retiring for 10 full days after which the dosage was cut to 250,000 units per day given just before retiring. This continued for the next 24 days. Thus, the second and third samples of saliva were taken from recruits receiving oral penicillin continuously for 10 days and 31 days, respectively. At the time of the beginning of these tests the incidence of Group A streptococcus within the regiment from which this group was selected was 14 per cent.[3]

The hyaluronidase determinations were completed on each sample of saliva using the viscosity reduction technic.[1] All results were recorded in seconds' decrease in viscosity of saliva-hyaluronic acid substrate mixture. The average of all tests conducted for each group was selected for correlation.

RESULTS

The average hyaluronidase activity for the first group prior to the administration of penicillin was 9.8 seconds reduction in viscosity (Table I). At this time the incidence of Group A streptococcus in the regiment from which this company was selected was as high as 13 per cent, and the streptococcal upper respiratory disease rate was 18 cases per thousand, per week. At the time of the tests, all subjects sampled were apparently symptom free or they would not have appeared for the saliva collection.

Three days after the penicillin injection the average hyaluronidase activity of the group dropped to 7.6 seconds during which time the incidence of Group A streptococcus in the regiment dropped to 2 per cent, and the rate of disease to 1.5 cases per thousand per week.

The third week (14 days after the initial test and 10 days after the injection of penicillin), the hyaluronidase activity of saliva returned to 10.3 seconds, during which time the Group A incidence in the regiment returned to 15 per cent, and the rate of upper respiratory infection returned to 20.0 cases per thousand per week.

The changes in hyaluronidase activity were more marked in the subjects receiving the larger dosage of penicillin. However, the reaction was apparent in the group receiving 600,000 units also and are, therefore, summarized as one group. The times for these hyaluronidase determinations were January 25, February 1, and February 7, 1955, respectively.

*Cilloral Tablets, Bristol Laboratories, Inc.

TABLE I

HYALURONIDASE ACTIVITY OF SALIVA, INCIDENCE OF GROUP A STREPTOCOCCUS AND RATE OF UPPER RESPIRATORY INFECTION OF 56 YOUNG MALE ADULTS BEFORE AND AFTER INTRAMUSCULAR PENICILLIN THERAPY
(USNTC BAINBRIDGE, MD., JANUARY AND FEBRUARY, 1955)

PENICILLIN THERAPY*	DAYS INTERVAL	AVERAGE HYALURONIDASE ACTIVITY†	PER CENT OF TOTAL INCIDENCE OF GROUP A STREPTOCOCCUS‡	STREPTOCOCCAL UPPER RESPIRATORY INFECTION§
None	0	9.8	13.0	18.7‖
3 days after penicillin	7	7.6	2.0	1.5
10 days after penicillin	14	10.3	15.0	20.0

Source (for data on streptococcus): Anderson, H. C., and Schreier, A. J.: A Summary of Studies on Streptococcal Disease: Incidence, Treatment and Prevention at USNTC, Bainbridge, Md., 1954-55, Report issued by the Medical Department, USNTC Bainbridge, 1955.

*One intramuscular dose of penicillin, 600,000 units to 900,000 units.

†Recorded in seconds drop in viscosity of saliva substrate mixture per 45 minutes at 37° C.

‡Determined from random throat culture surveys (25 per cent) of regiment in which selected company was located.

§Determined from number of individuals reporting to sick call per thousand per week from regiment in which selected company was located.

‖Rate in regiment before penicillin therapy, but also before the selected group entered the regiment—not strictly comparable to other data.

The average hyaluronidase activity for the second group prior to the administration of penicillin was 11.3 seconds decrease in viscosity (Table II). At this time the incidence of Group A streptococcus in the regiment from which this company was selected was as high as 14 per cent, and the disease rate per thousand was 10.3 cases per week. At the time of the tests all subjects sampled were apparently symptom free or they would not have appeared for the saliva collection.

After 10 days of 50,000 units oral penicillin per day the average hyaluronidase activity of the group dropped to 8.0 seconds.

TABLE II

HYALURONIDASE ACTIVITY OF SALIVA, INCIDENCE OF GROUP A STREPTOCOCCUS AND RATE OF UPPER RESPIRATORY INFECTION OF 49 YOUNG MALE ADULTS BEFORE AND AFTER ORAL PENICILLIN THERAPY
(USNTC BAINBRIDGE, FEBRUARY AND MARCH, 1955)

PENICILLIN THERAPY*	DAYS INTERVAL	AVERAGE HYALURONIDASE ACTIVITY†	PER CENT OF TOTAL INCIDENCE OF GROUP A STREPTOCOCCUS‡	STREPTOCOCCAL UPPER RESPIRATORY INFECTIONS§
None	0	11.3	14.0	10.3
10 days of oral penicillin	14	8.0	2.0	5.0
31 days of oral penicillin	35	6.0	0.5	0.6

Source (for data on streptococcus): Anderson, H. C., and Schreier, A. J.: A Summary of Studies on Streptococcal Disease: Incidence, Treatment and Prevention at U. S. Naval Training Center, Bainbridge, Md., 1954-55, Report issued by the Medical Department, USNTC Bainbridge, 1955.

*Oral penicillin 500,000 units per day for the first 10 days, 250,000 units per day for the next 24 days.

†Recorded in seconds drop in viscosity of saliva substrate mixture per 45 minutes at 37°C.

‡Determined from random throat culture surveys (25 per cent) of regiment in which selected company was located.

§Determined from number of individuals reporting to sick call per thousand per week from the regiment in which selected company was located.

Thirty-five days after the first saliva analysis and 31 days after continuous penicillin therapy the hyaluronidase activity dropped to 6.0 seconds. During this time the incidence of Group A streptococcus as indicated by random testing of the regiment dropped to 2 per cent and finally to 0.5 per cent. Similarly the streptococcal disease rate dropped to 0.6 cases per thousand per week.

The times of these tests were February 14 and 28 and March 21, 1955, respectively.

DISCUSSION

It is apparent that a significant drop in the hyaluronidase activity of saliva occurred after the injection of penicillin and that 10 days after the injection the hyaluronidase activity returned to approximately its original value. This change was similar to the change that occurred in the incidence of Group A streptococcus and the rate of streptococcal upper respiratory infections in the regiment within which the test group was located.

It is also apparent that the hyaluronidase activity of saliva dropped after treatment with oral penicillin. This change was similar to the change that occurred in the incidence of Group A streptococcus and the rate of streptococcal upper respiratory infections found in the regiment within which the test group was located.

Inasmuch as the hyaluronidase activity of saliva has been demonstrated to be of bacterial origin,[4] it is logical to expect a change as a result of antibiotic therapy. It is interesting to note this change in relationship to the response of streptococcal disease rate and estimated incidence. Although the latter was reduced to a negligible amount, there was still a significant activity, although reduced, of salivary hyaluronidase. This could be accounted for by the following reasons: (1) all of the microorganisms capable of producing hyaluronidase were not sensitive to the antibiotics, (2) the antibiotic dosage or method of administration was not effective for the flora of the oral cavity, or (3) the incidence of Group A streptococcus in the regiment as determined from throat swabbings of 25 per cent of the personnel and rate of upper respiratory disease had little relationship to the oral microflora.

CONCLUSIONS

1. Salivary hyaluronidase activity of young male adults decreases after intramuscular administration of penicillin and after oral penicillin therapy.

2. Salivary hyaluronidase activity of young male adults responded to the antibiotic in a manner similar to the response of Group A streptococcus as determined by random throat culture surveys and the rate of streptococcal upper respiratory infections of recruits in the regiments from which the test groups were selected.

3. In spite of the prolonged oral penicillin therapy, salivary hyaluronidase activity of naval recruits was not reduced to negligible activity, in most instances.

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TOPOGRAPHIC SAMPLING METHOD FOR QUANTITATIVE RADIOTRACER EVALUATION IN BONES AND TEETH

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THE extent of radioisotope uptake in bones and teeth generally is evaluated in 2 manners: (1) radioautographic representation, a qualitative and semiquantitative expression of the localization of the radioactive element within the tissue and (2) specific activity, a quantitative measure of the average uptake in a tissue sample. Both methods of evaluation have certain distinctive limitations. Radioautography is restricted in its ability to reveal the full range of uptake when the radiotracer of a bone-seeking and tooth-building element varies greatly in distribution from one region of a calcified tissue to a closely adjacent area. On the other hand, specific activity determinations on whole tissue samples are not predictive of any gradient within that tissue. Hence the value of specific activity determinations can be increased in proportion to the degree of accuracy with which increasingly minute samples can be prepared from the tissue under investigation. Where large gradients in isotope uptake exist within a mineralized tissue, a selective grinding method for the preparation of minute and carefully oriented samples, on which specific activity determinations may be made, has been found to be of value in the evaluation of these gradients. This method was employed first in studies on the uptake of radiophosphorus in the enamel of cat and dog teeth[1] and has recently been applied effectively in the preparation of samples for analysis of the trace element distribution in human teeth from different age groups.[2]

In recent radiotracer studies on bones and teeth of rhesus monkeys, the selective grinding method has been developed further in order to obtain several consecutive layers from selected areas of enamel, dentin, cementum, and long and flat bones.[3, 4] In this report, 2 experiments, typical of studies on radiophosphorus and radiosodium distribution in the rhesus monkey, will be described, in which the preparation of discrete and distinct topographic layers from various calcified tissues has been tested to determine the degree of reproducibility of the results from tooth to tooth and from bone to bone. In addition, the results of an experiment with radiosodium to test the ability to adapt the selective grinding method for use in rodents will be reported.

GENERAL PROCEDURE

Each tooth or bone that was to be sampled by the selective grinding procedure was mounted by means of sticky dental wax in a shell vial in such a

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position that the surface or surfaces from which samples were to be prepared were readily accessible (Fig. 1). Successive layers were ground from the surface inward during constant inspection of the process at a magnification of 10 diameters with the aid of a binocular dissecting microscope. Enamel samples were selectively ground from the tooth with small diamond stones (Tiffany 6D) attached to a dental handpiece in a standard dental engine; dentin and bone samples were prepared comparably with dental steel fissure burs (Revelation Bur No. 557 for straight handpiece). During the grinding process, the diamond stones or steel burs were moved back and forth over the tissue surface until a layer of uniform depth had been removed from an appropriately selected area. The grinding procedure was done under a chemical hood with appropriate protection against contamination. From 5 to 10 mg. of powder were collected from each layer and transferred to a small piece of black photographic paper for easier viewing. Each powdered sample was then weighed, and the radioactivity in the sample determined with a Tracerlab Autoscaler. Radioactivity uptake was recorded as per cent injected dose per gram of powdered sample. Finally, total phosphorus was determined for each sample of bone, according to the method of Fiske and Subbarow.[5] Specific details for application to the 2 species studied, monkey and rat, are described more advantageously in the following experiments which are illustrative applications of the method.

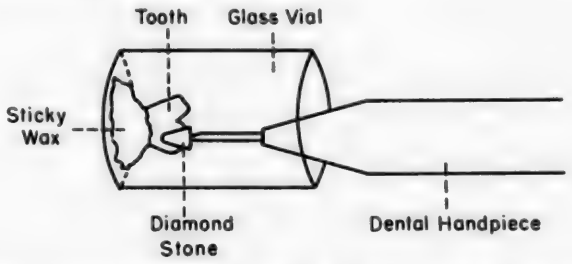

Fig. 1.—Diagram illustrating the mounting of a molar tooth for selective grinding.

EXAMPLES OF APPLICATION

Monkeys.—Two young female rhesus monkeys of approximately 3 Kg. were used in the 2 experiments which are to be described. The first animal received intravenously 10 mc. of radiophosphorus in the form of $NaHP^{32}O_4$ in isotonic saline solution. In this animal, the following permanent teeth were fully erupted in all 4 jaw quadrants: first and second incisors and first molars. Both upper second molars were unerupted; the lower second molars had emerged to a point where the mesial half was visible. Five minutes after the radiophosphorus administration, a calculated overdose of Nembutal was given to the animal. The second monkey was injected with 20 mc. of radiosodium as $Na^{24}Cl$ in isotonic solution. This animal was somewhat younger. Only the first incisors and first molars were fully erupted and functioning in all quadrants, whereas the second incisors had partially erupted and the second molars

were unerupted. Twenty-four hours after injection, this animal was sacrificed by an overdose of Nembutal. Immediately after sacrifice, the 4 first incisors, the 4 first and the 4 second molars of each animal were extracted and appropriate portions of the calvarium and the alveolar bone, and the whole femur and humerus were dissected. The long bones were split along their longitudinal axes and the bone marrow removed as thoroughly as possible. In these studies only the shaft was used. Each bone and tooth was wiped clean of adhering fluid, whether saliva or blood, and debris, and mounted in individual shell vials as described above.

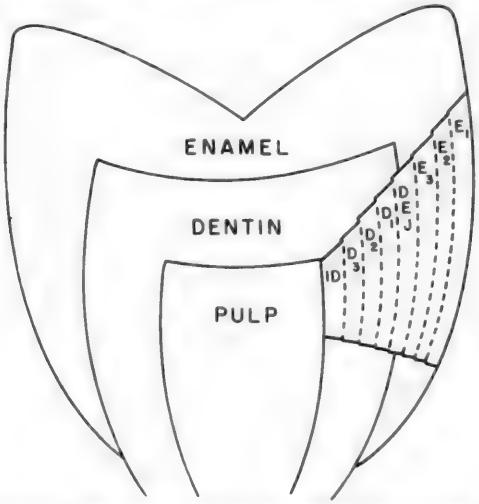

Fig. 2.—Diagrammatic representation of the appropriate location of the various layers of molar (buccal) enamel and dentin removed by selective grinding. ($E_{1, 2, 3}$—enamel; *DEJ*—dentinoenamel junction; $D_{1, 2, 3}$—dentin; *ID*—internal dentin.)

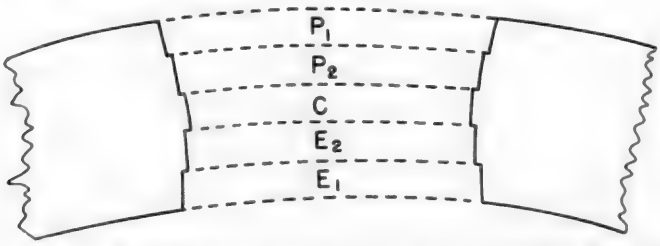

Fig. 3.—Diagram of a longitudinal section of long bone illustrating the location of the 5 layers from which samples were removed by selective grinding. ($P_{1, 2}$—subperiosteum; *C*—center; $E_{1, 2}$—endosteum.)

From the buccal surface of each tooth, 8 successive powdered samples were prepared as shown in Fig. 2: 3 of the enamel proper (E_1, E_2, E_3), 1 in the region of the dentinoenamel junction (DEJ), and 4 dentin samples (D_1, D_2, D_3, ID). Each layer removed was approximately 0.3 mm. thick; the area sampled was a square or oblong about 3 mm. high in the cervico-occlusal dimension and 3 to 6 mm. wide in the mesiodistal dimension. The depth and uniformity of each layer was gauged by beginning each successive layer

slightly inside the perimeter of the previous one in a steplike fashion (Fig. 2). The margins of the buccal surface adjacent to the gingiva and to the occlusal surface were avoided in the sampling process. Powdered samples from a maximum of 5 layers were prepared from each of the calvarium, alveolar bone, and shaft of the femur and humerus. The samples were ground in the following order as illustrated in Fig. 3: Subperiosteal layers (P_1 and P_2), endosteal layers (E_1 and E_2), and central layer. A maximum of 3 samples of root cementum were obtained: samples from the layer closest to the periodontal membrane (subperiodontal membrane layer C_1), the central cementum layer (C_2), and the layer adjacent to the root dentin (peridentin C_3) which unavoidably contained some root dentin.

Radiophosphorus.—The data for the first monkey experiment expressed in terms of the per cent of the injected dose of radiophosphorus incorporated per gram of powdered sample of enamel and dentin of monkey teeth are recorded in Table I. Samples taken from similar layers of bilaterally symmetrical teeth in the same dental arch and in the same state of eruption and function contained fairly comparable quantities of radiophosphorus even after this short time interval. For example, comparable layers in the unerupted upper right and left second molars were within the same range although not absolutely identical in uptake. Where the teeth in both arches were completely erupted and functioning, as were the 4 first molars, spatially comparable layers in each of the 4 teeth gave results of the same order. Thus,

TABLE I

THE UPTAKE OF RADIOPHOSPHORUS IN THE FIRST AND SECOND PERMANENT MOLARS OF THE RHESUS MONKEY*

	FIRST MOLAR				SECOND MOLAR			
	ERUPTED AND FUNCTIONING				PARTIALLY ERUPTED		UNERUPTED	
	LR	LL	UR	UL	LR	LL	UR	UL
Enamel								
Surface layer (E_1)	4	5	6	5	243	178	519	340
Second layer (E_2)	4	2	1	5	29	31	63	60
Third layer (E_3)	5	4	2	2	31	6	39	26
Dentinoenamel junction (DEJ)	7	5	4	3	23	22	67	55
Dentin								
Outer layer (D_1)	15	14	14	8	5	8	4	12
Second layer (D_2)	21	23	17	12	11	8	15	18
Inner layer (D_3)	59	31	27	18	18	15	33	29
Internal dentin (ID)	52	44	47	52	67	65	107	116

*Per cent injected dose per gram of powdered sample $\times 10^{-4}$.

the comparative uptakes in per cent of injected dose per gram in the surface layer (E_1) for these teeth were 4×10^{-4}, 5×10^{-4}, 6×10^{-4}, and 5×10^{-4}, respectively, while the uptakes in internal dentin (ID) were 52×10^{-4}, 44×10^{-4}, 47×10^{-4}, and 52×10^{-4}, respectively. In the case of the second molars, the lower teeth were in a more advanced state of development and eruption than were the uppers. This difference was reflected in the greater amount of radiophosphorus in all layers of the upper teeth as compared with that in comparable areas in the lower molars.

The total phosphorus content in the samples from the various layers of long bone shaft was fairly uniform and averaged 11.5 per cent, except for the subperiosteal layer which was 10.5 per cent. The radiophosphorus concentration in the right femur and humerus shaft layers expressed in terms of specific activity in contrast to per cent uptake for the teeth in the preceding paragraph agreed reasonably well with that present in similar layers of the left long bones (Table II). For example, the specific activity of the external

TABLE II

THE SPECIFIC ACTIVITY OF TOTAL PHOSPHORUS IN BONE AND CEMENTUM OF THE RHESUS MONKEY*

	SUB-PERIOSTEAL LAYER P_1	SUB-PERIOSTEAL LAYER P_2	CENTRAL LAYER	ENDOSTEAL LAYER E_2	ENDOSTEAL LAYER E_1
Humerus Shaft					
Right	1,820	200	90	75	230
Left	1,800	150	215	325	295
Femur Shaft					
Right	875	70	70	105	345
Left	630	50	60	80	290
Alveolar Bone					
Upper					
Right	3,260	860	1,110	1,180	2,355
Left	3,400	1,195	1,250	1,470	2,330
Lower					
Right	1,930	460	1,030	1,040	2,020
Left	1,830	1,400	1,440	1,300	2,020

	SUBPERIODONTAL MEMBRANE LAYER C_1	CENTRAL LAYER C_2	PERIDENTIN LAYER C_3
Root Cementum			
Upper			
Right	2,200	204	88
Left	1,909	86	62
Lower			
Right	1,310	114	66
Left	1,025	63	67

*All values are $\times 10^{-3}$.

layer of right humerus shaft was $1,820 \times 10^{-3}$ while that of the left humerus was $1,800 \times 10^{-3}$. The concentration of radiophosphorus in the various layers of the humerus was approximately double that of comparable layers in the femur, except for the endosteal layers which had taken up relatively similar quantities of radiophosphorus in both femur and humerus. In alveolar bone, the uptake in the maxillary subperiosteal layer was almost twice that of the mandible but again each sample taken from bilaterally symmetrical portions within the same arch gave comparable results, as evidenced for example by the specific activity of the external layer of the upper right alveolar bone which was $3,260 \times 10^{-3}$ while that of the upper left alveolar bone was $3,400 \times 10^{-3}$.

Radiosodium.—The concentration of radiosodium within the layers of enamel and dentin of the second monkey are expressed in Table III as per

cent injected dose per gram of sample. As with radiophosphorus, when the 4 teeth were in the same state of eruption and function, there was a fairly good agreement between comparable layers. This can readily be seen in the values 78×10^{-4}, 87×10^{-4}, 83×10^{-4}, 78×10^{-4} per cent, respectively, in the surface layers (E_1) of the 4 teeth; 68×10^{-4}, 68×10^{-4}, 60×10^{-4}, 78×10^{-4} per cent, respectively, in the second layer (E_2); etc. The extent of difference between the uptake of radiosodium in unerupted and erupted teeth was not as great as with radiophosphorus, but was nevertheless significant.

TABLE III

THE UPTAKE OF RADIOSODIUM IN THE FIRST AND SECOND PERMANENT MOLARS OF THE RHESUS MONKEY*

	FIRST MOLAR				SECOND MOLAR			
	ERUPTED AND FUNCTIONING				UNERUPTED			
	LR	LL_2	UR	UL	LR	LL	UR	UL
Enamel								
Surface layer (E_1)	78	87	83	78	141	140	147	163
Second layer (E_2)	68	68	60	75	101	108	119	110
Third layer (E_3)	85	112	69	105	75	83	108	115
Dentinoenamel junction (DEJ)	152	159	168	230	103	115	92	137
Dentin								
Outer layer (D_1)	569	510	618	485	861	592	564	472
Second layer (D_2)	509	530	435	706	849	1,009	784	704
Inner layer (D_3)	633	644	560	682	1,035	1,058	976	864
Internal dentin (ID)	781	706	389	1,441	1,285	1,280	745	1,070

*Per cent injected dose per gram of powdered sample $\times 10^{-4}$.

Discussion.—The specific advantage achieved by the selective grinding method is the ability to prepare numerous minute, discrete samples from topographic areas of a calcified tissue. This separation is especially useful where large gradients of radioactive uptake exist within a block of calcified tissue. A typical example is noted in the enamel of the upper right second molars in Table I, where the range of uptake varied from 39×10^{-4} per cent in the third enamel layer sample (E_3) to 519×10^{-4} per cent in the enamel surface sample (E_1) in a distance of about 1 mm. of tooth substance. If enamel in this case had been separated from dentin by the bromoform flotation-centrifugation procedure, this gradient would have been obscured in the single average specific activity value for all enamel. A similar situation would have occurred for dentin where the samples prepared by selective grinding from the same tooth indicated a difference in uptake, varying from 4×10^{-4} per cent in outer dentin (D_1) to 107×10^{-4} per cent in internal dentin (ID). On the other hand, if a radioautographic representation had been attempted, these intense differences would have been demonstrable to some degree, but the full extent of the variation could not be delineated because of the wide range of activity within closely adjacent areas. Along with this ability to evaluate gradients, there is clear evidence that the layers into which the teeth or bones have been separated were relatively reproducible from tooth to tooth and from bone to bone as seen in Tables I, II and III.

When larger animals such as primates (e.g., rhesus monkeys) are used in the study of mineralized tissues, adequate experimental controls as to age, sex, diet, hereditary background, dental development, etc., are presently impossible to achieve. As a result, it is desirable, whenever possible, to have the animal serve as its own control. In the case of dental tissues where there is a duplicity of structure, either one of the symmetrical pairs of teeth could be used as the control for the other, if both reacted similarly under similar conditions. Thus, teeth from the opposite quadrant could be taken for samples at different time intervals after radioisotope administrations. The results of the reported experiments indicate that this is feasible with bilaterally symmetrical teeth as seen in Table I and Table III. Where the teeth in the 4 quadrants are in the same state of function and development (e.g., fully erupted and functioning), 1 tooth may be used as the control for the other 3 of the same type. Thus, the teeth of 1 quadrant may be removed at one time interval after administration of the radiotracer; those of the second, third, and fourth quadrants can be extracted at 3 later times, respectively, for comparisons of uptake at four time intervals. Other variables than time may be superimposed in like fashion. This internal control method has been used successfully with radiophosphorus[2] and in experiments with other radiotracers to be reported elsewhere.

At the short time interval used in the radiophosphorus experiment, the specific activity of the subperiosteal portion of maxillary alveolar bone was greater than that in the mandibular bone. This difference may be in part the result of a greater porosity of maxillary bone and a greater vascularity. Therefore, comparison of radioisotope uptake in alveolar bone at two different time intervals is better made between samples taken from the same arch.

Rats.—Five 100 Gm. rats were each injected subcutaneously with 600 μc. radiosodium as $Na^{24}Cl$ in isotonic solution and sacrificed 24 hours later. Heart-blood samples were withdrawn immediately prior to sacrifice. The 4 quadrants of rat jaw with the 3 molars of each quadrant in situ were dissected out; all soft tissues were removed. The surface of the teeth was gently wiped free of any adhering saliva. Debris lodged in the fissures were removed with a 20-gauge syringe needle. Each quadrant was then mounted in a shell vial. Only 1 sample of enamel and 1 of dentin could be obtained from each quadrant because of the small size of the rat molar and the thinness of the enamel and dentin. For the very narrow fissure surfaces of enamel, a Tiffany 2A diamond stone was used, whereas a larger Tiffany 2D+ stone sufficed for the bulk of enamel. The total amount of enamel sample that could be obtained from 1 quadrant was approximately 8 mg. when both the buccal and lingual surfaces of 3 molars, the mesial surface of the first molar, and the distal surface of the third molar were used. Because of the exposed dentin on the cusps and the likelihood of retention of radioactive saliva and food debris in the sulci, the occlusal surface was not used. Only one layer of bone shaft, free of marrow, could be obtained because of the thinness of the rodent bone.

The concentrations of radiosodium in terms of per cent uptake of injected dose per gram of the various mineralized tissues and per milliliter of serum of the rat are tabulated in Table IV. The results obtained from the composite sample from the 3 molars in each of the 4 quadrants were within 6 per cent of the mean for the 4 quadrants of each animal. A typical variation of the results in per cent uptake by the molar enamel of the 4 quadrants of 1 animal was as follows: 1.91; 1.99; 1.94; 1.81. Furthermore, the ratios of the radiosodium concentration in the various mineralized tissues to the concentration in the serum were in remarkably good agreement between animals for each of the mineralized tissues examined. The relative uptake in bone was slightly higher than it was for dentin, which, in turn, was only 20 per cent higher than was found in enamel. This relatively high concentration of radiosodium in rat enamel as compared to dentin was a striking contrast to the relatively low concentration in enamel observed in monkeys.

TABLE IV

THE UPTAKE OF RADIOSODIUM IN THE ENAMEL, DENTIN, FEMUR, AND BLOOD OF THE RAT

ANIMAL	MOLAR ENAMEL*				MOLAR DENTIN*				FEMUR SHAFT*	BLOOD SERUM†
	LR	LL	UR	UL	LR	LL	UR	UL		
A	1.91	1.99	1.94	1.81	2.56	2.53	2.66	2.60	2.67	2.50
B	2.18	2.12	1.85	2.18	2.72	2.75	2.40	2.64	2.76	2.81
C	1.60	1.50	1.61	1.58	2.10	2.23	1.92	1.85	2.43	2.34
D	2.35	2.23	2.40	2.14	2.98	2.54	2.87	3.07	3.04	3.17
E	2.13	1.99	1.93	2.00	2.90	2.47	2.33	2.31	2.51	2.56

*Expressed as per cent of injected dose per gram of calcified tissue.
†Expressed as per cent of injected dose per milliliter of blood serum.

Discussion.—The use of mineralized tissues of rats presented problems of a different nature than those encountered in monkeys. Whereas 3 distinct layers of enamel and 4 of dentin could be obtained from 1 tooth of a monkey, it was technically extremely difficult to remove more than 1 layer each of the enamel and dentin pooled from the 3 molars of a jaw quadrant. Hence, the rat could not be used as its own control as in the fashion described for the monkey. On the other hand, it was possible to have a larger number of animals and adequate littermate controls. As seen in Table IV, there was very good agreement in the uptake of isotope in the enamel and dentin of different quadrants of the same animal. The close agreement between different animals of the same age was a further demonstration of the usefulness of the method. Because the rat has now become an important experimental animal in dental research, the possibility of adapting the grinding method for use in rats allows for further study of the mineralized tissues in this species.

However, any generalization of data from one species to others must be done with caution. For example, the difference between uptake in monkey enamel as compared to rat was considerable, yet consistent. This difference may be related to the greater density of monkey enamel than that of the rat, as has been suggested by specific gravity measurements.[6]

SUMMARY

A selective grinding method for sampling bones and teeth of rhesus monkeys and rats has been described and found to be especially useful where the distribution of radioisotope varied greatly from one region of calcified tissue to another. The method was evaluated following radiophosphorus and radiosodium injection in monkeys in order to determine whether reproducible results could be obtained between bilaterally symmetrical teeth and between various bones.

In monkeys, good reproducibility was observed in both radiophosphorus uptake and total phosphorus content between bilaterally symmetrical teeth within the same arch, and in different samples of alveolar bone and cementum of the same arch. Comparable radiophosphorus uptakes were observed between the shafts of the right and left humeri of the same animal and also between the shafts of the right and left femurs. Similarly, there was good agreement in radiosodium uptake between teeth that were in the same state of development and function.

In rats that received subcutaneous radiosodium injections 24 hours before sacrifice, reproducible results were obtained in the relative uptake of isotope in enamel and dentin from the 4 jaw quadrants within each animal and also between animals of the same litter. Comparable results were obtained in the comparison of the right and left femurs.

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ERUPTIVE MECHANISM AND MOVEMENT IN THE FIRST MOLAR OF THE RAT

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ERUPTION is essentially the movement of a tooth or a tooth germ as related to its bony crypt and its overlying mucosa. To study the direction or mechanism of eruption, therefore, one must (1) establish some fixed points of reference within the jaws, and (2) compare the movement of the tooth at successive stages. Under normal circumstances, however, bone formation and bone destruction occur simultaneously and no permanent landmark remains in a growing bone. Thus, a study concerning the eruptive movements of teeth is difficult. Greep[1] discovered a recessive mutation in rats *(ia)* which is characterized by a retardation or absence of bone resorption but in which bone apposition occurs in a more or less normal manner.[2, 3] Consequently, if one were to compare the bones of these animals at successive stages, the growth pattern of these bones becomes apparent. This method has been used to study the detailed growth patterns of various bones.[3-6]

Since the *ia* bones present numerous fixed points of reference, the jaws in these animals offer an unusual opportunity to study the course and mechanism of eruption. In the present investigation, therefore, an attempt was made to determine the cause as well as the direction of movement of the mandibular first molar in the rat. In a part of this study, the *ia* and normal animals were used, while in part it depends exclusively on the normal rats.

METHODS AND MATERIALS

The investigation is based on serial histologic sections through the first molar region of 20 *ia* and 34 heterozygous rats, ranging from 13 days' insemination age to 30 days after birth. The heads were fixed in 5 per cent formalin, decalcified in 5 per cent nitric acid, embedded in celloidin, cut serially in the frontal plane and stained with hematoxylin and eosin.

Camera lucida tracings were made of representative areas in all the *ia* and normal mandibles. Using various fixed points in the mandible[4] these tracings were then superimposed in the following manner: (a) *ia* tracings of successive stages were superimposed to determine the direction and extent of tooth movement; (b) *ia* and normal tracings of identical stages were superimposed to

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determine the role of bone resorption in tooth movement; and (c) since from 13 days' insemination age to 5 days after birth the *ia* first molar tooth germ is relatively undisturbed, up to this stage the *ia* jaws could be used to advantage. From 5 days to 30 days after birth, tracing of successive normal jaws were superimposed to determine the extent and direction of movement. In part, this study consists of a histologic analysis of the eruptive changes in and around the first molar tooth germ.

REVIEW OF LITERATURE

Literature concerning the eruptive movement of teeth is voluminous and contradictory. Since in different studies different aspects of eruption have been emphasized, the information on this subject may be grouped under the following main headings.

A. *Movement of the Tooth Germ and Tooth.*—The movement of developing teeth prior to the formation of the root was first recognized by Brash.[7] Brodie[8] suggested that prior to eruption the developing and growing teeth move to adjust their position in the growing jaws. Weinmann[9, 10] reported that during the pre-eruptive phase (prior to the beginning of root formation) the tooth germs move bodily as well as by excentric growth. These movements are associated with apposition in the area of the crypt from which the tooth migrates and resorption of bone on the side of migration. In contrast, Landsberger,[11] Diamond and Applebaum,[12] and Diamond,[13] reported that the developing crown did not leave its original initiation site until root formation began and that its vertical height increased by shifting of cervical loops in the direction of the fundus. According to these authors, the only occlusal displacement prior to root formation was the addition of enamel increments. Sicher[14, 15] proposed that the developing root was prevented from growing into the alveolus by the hammock ligament and that the epithelial diaphragm was a fixed point.

The continuous eruption of human teeth is recognized and has been subdivided into the active and passive phases.[17, 18, 19]

B. *Changes in the Crypt.*—That the crypt undergoes resorption during the growth of the tooth germ has been shown repeatedly.[9, 20, 21] During the stage following the start of root formation, bone apposition occurs rapidly at the fundus of the crypt.[9, 16] The apposition and resorption pattern of bone in the crypt has been related to the rate of growth and the direction of eruption of the tooth.[4] In addition, Brash[7] showed, in madder-fed pigs, that during the active eruption extensive growth is seen on the crests of alveolar crypts.

C. *Tooth Movement and Growth of Alveolar Process.*—Landsberger[11] found that the removal of tooth germs from the maxilla of dogs led to an absence of the alveolar process, shortening of the maxilla, and failure of the floor of the nose to descend normally. Further, it has been shown that in cases of anodontia, although the mandible develops normally, its alveolar process is completely missing.[22, 23]

D. *Tooth Eruption and the Follicle.*—Orban[24] described the primitive periodontal membrane as having 3 distinct layers, 2 outer layers adjacent to the bone and the tooth germ, respectively, and a central zone. The outer zones

consist of collagenous fibers, while the intermediate zone consists mainly of precollagenous fibers. During active rapid eruption, the intermediate layer or plexus permits the continuous rebuilding and rearrangement of the periodontal fibers. In the region of the fundus, according to Sicher,[15] the dental follicle differentiates into 2 layers: one close to bone consisting of loose connective tissue and the other a network of thick fibers adjacent to the growing end of the tooth. The latter layer contains a large amount of fluid in the tissue spaces and was named the hammock ligament.[15]

E. *Mechanism of Tooth Eruption.*—Theories on the mechanism of eruption are numerous. A critical review of various concepts concerning the force underlying eruption was given by Massler and Schour.[25] After a detailed evaluation, it was concluded that the evidence, though inconclusive, favored the view that the vascularity of the tissues was the source of the eruptive force. Sicher[14, 15] suggested that the eruptive mechanism is the proliferation of the pulp coordinated with the growth of Hertwig's epithelial sheath. The force generated by proliferating pulp, acting against the hammock ligament, causes the tooth to migrate into the oral cavity. After the tooth reaches the occlusal plane and the root is completed, it is the differential growth of the bone and the cementum which causes the continued eruption of the tooth. In the multirooted teeth, there is no hammock ligament and it is the apposition at the inter-radicular septum which causes the tooth to erupt.

FINDINGS

A. *Fifteen Days' Insemination Age to 5 Days After Birth.*—

1. *Tooth and crypt development:* Between 15 and 16 days' insemination age, the ossification of the mandible began. At 17 days' insemination age, the first molar region of the mandible was in the form of a bony trough, the upper part of which contained the tooth bud and the lower the inferior dental nerve. At 20 days' insemination age, by the formation of a transverse plate of bone the inferior dental nerve was separated from the molar tooth germ. This plate of bone thus completed the formation of the molar crypt.

In the interval between 16 and 20 days' insemination age, the tooth germ continued to enlarge. At 20 days' insemination age, the membrana preformativa appeared. Between 20 and 21 days' insemination age, dentin formation began. At 1 day after birth, the dentin started to calcify, and at 3 days the formation of enamel began.

2. *Movement of the tooth germ:* Tracings of frontal sections, through the middle of the *ia* and normal first molar tooth germ at similar developmental stages, were superposed. These comparisons revealed that up to 5 days after birth, the position of the *ia* first molar tooth germ, in relation to the oral epithelium and the jaw was similar to that of the normal.

ia rats: From initiation to 19 days' insemination age, the *ia* first molar tooth germ was in the cap stage of development. At 20 days' insemination age, when the tooth germ assumed a bell shape, the growth of the lingual cervical loop of the enamel organ was more rapid than the buccal, with the result that

the tooth germ assumed a tilted position within the crypt. This position was such that the future occlusal plane of the tooth was inclined inward and upward and the cervical opening was directed downward and outward (Fig. 1).

From 20 days' insemination age to 5 days after birth, the *ia* first molar grew considerably in size but maintained this inclination. A superposition of comparable tracings of the *ia* mandible at successive stages, indicated that the dentinoenamel junction progressively shifts upward, and that the most important shift of the tooth germ was in a superolateral direction as indicated by the arrows in Figs. 2, 3, and 4. During this period, although there was an increase in size of the *ia* tooth germ, there was no distortion of its morphology except in the area of the cervical loops. The fact that in the *ia* rats, despite

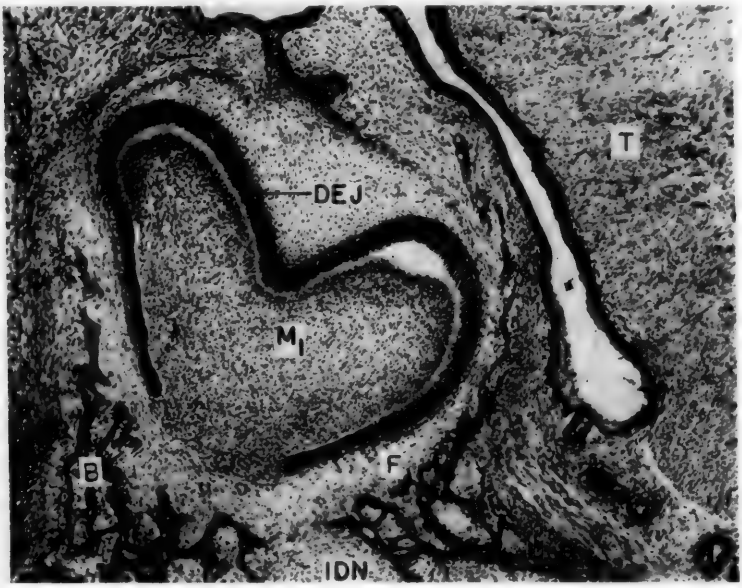


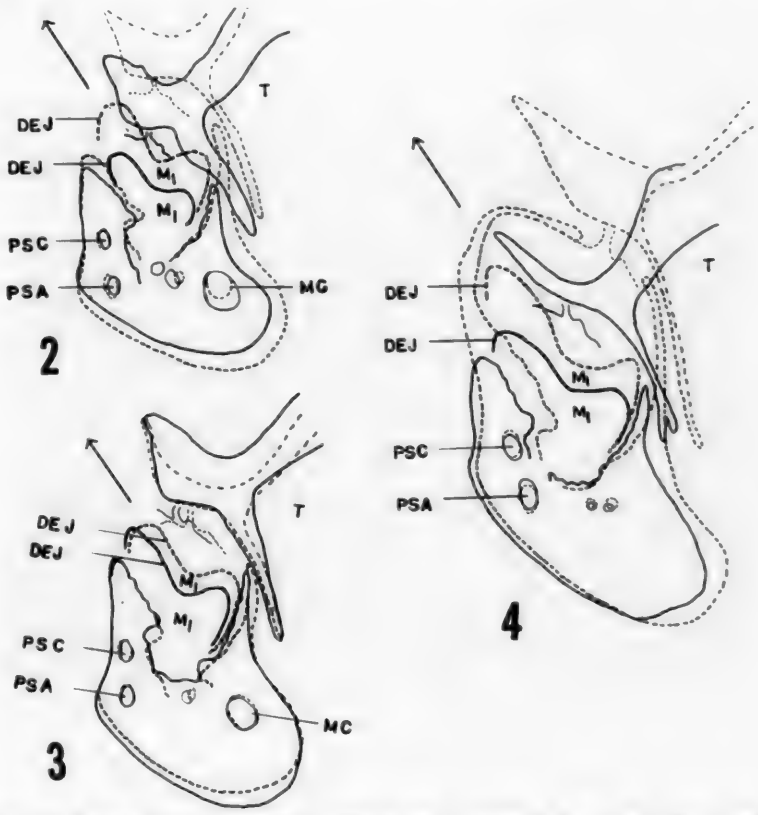
Fig. 1.—First molar tooth germ in normal rat 21 days' insemination age. *T*—tongue; *DEJ*—future dentinoenamel junction; *M*—first molar; *F*—follicle; *IDN*—inferior dental canal; *B*—bone. (Orig. mag. ×80.)

the absence of bone resorption, the molar tooth germs enlarge without much distortion, further indicates that these tooth germs move bodily away from the bottom of the crypt, i.e., they move progressively out of the jaws.

Although up to 5 days of age, the coronal part of the tooth germ showed no distortion, folding of the cervical loops occurred as early as 19 days' insemination age. This folding was due to the impingement of bone trabeculae of the crypt on the growing cervical loops. This fact indicated that in addition to the bodily movement of the tooth germs out of the jaws, their cervical part also grew deeper into the jaws.

Camera lucida drawings of the alveolar process in the area of the *ia* first molars, at successive stages, showed that its height progressively increased from 17 days' insemination age to 5 days after birth. The direction of growth of the

alveolar process, as seen by the arrangement of the bony plates, was upward and outward. However, the tooth germ maintained a constant relationship with the medial and lateral alveolar crests, indicating once again its upward and outward migration. Since the alveolar crest was always below the widest part of the tooth germ (Fig. 1), it is obvious that the upward and outward migration of the tooth germ was faster than the growth of the crest. If the growth of the alveolar process had occurred independently, or prior to the upward bodily movement of the tooth germ, the latter would have shown gross distortion of shape.



Figs. 2, 3, and 4.—Superimposed camera lucida tracings of the first molar area in *ia* animals at successive stages to show an occlusal and lateral migration of the tooth germ. Broken line is the older stage. Fig. 2 represents 20 days' insemination age and birth. Fig. 3. 1 and 2 days after birth, and Fig. 4 represents 1 and 5 days after birth. *T*—tongue; M_1—molar tooth germ; *DEJ*—future dentinoenamel junction; *PSC*—perichondral splint of condylar cartilage; *PSA*—perichondral splint of angular cartilage. (Orig. mag. ×18.)

Normal rats: The superposed tracings of sections through first molar region of *ia* and normal rats at comparable stages, showed identical position of the teeth. The movements of the normal first molar tooth germ, therefore, were the same as described in the *ia* rats.

Study of serial sections of the bony crypt of the first molar tooth germ showed that, up to 20 days' insemination age, resorption occurred on all surfaces

of the crypt. From 21 days' insemination age to 5 days after birth, inactivity, or a varying degree of bone apposition, could be seen on the wall of the crypt which faced the inferolingual part of the tooth germ, while elsewhere bone resorption continued. The area of bone apposition was opposite to the direction in which the tooth germ was migrating.

3. *Changes in the follicle:* Up to 20 days' insemination age, the dental sac or follicle consisted of mesenchymal tissue with abundant inter-cellular fluid and stellate cells (Fig. 5). The width of the follicle was greater on the lingual and inferolingual side of the tooth germ than on the buccal (Fig. 5). From 21 days' insemination age to 3 days after birth, the follicle became much narrowed. It was predominantly cellular and consisted of fusiform cells which were arranged with their long axes parallel to the outer surface of the tooth germ.

Fig. 5.—First molar tooth germ (M_1) and mandible (B) at 20 days' insemination age. The follicle (F) in the inferolingual part of the tooth germ appears widest. T—tongue. (Orig. mag. ×80.)

The tissue did not contain much inter-cellular substance. On the buccal side, the follicle was compressed and fusiform cells were densely packed between bone and the outer enamel epithelium.

At 5 days, the width of the follicle was reduced further. However, it was still widest on the lingual side, where 6 to 7 layers of fusiform cells were arranged parallel to the surface of the tooth germ.

B. *Five Days to 8 Days After Birth.*—

1. *Tooth development:* The buccolingual width of the crown of the first molar was completed at 8 days after birth.

2. *Movement of the tooth germ:* (Normal rats.) From 5 to 8 days after birth, the first molar tooth germ maintained its inclination within the jaw. Its

occlusal surface still faced upward and inward, and its cervical surface faced downward and outward. At 5 days the relationship between the tooth germ and the alveolar crests changed. Whereas up to this time the tooth extended beyond the alveolar crests now the medial and lateral plates of the alveolar process surrounded it and extended to its occlusal surface. Thus the alveolar process, during this time, grew faster than the upward migration of the tooth germ.

The part of the crypt which faced the inferolingual surfaces of the tooth germ showed bone apposition, while elsewhere resorption of bone occurred. Bone resorption in the fundus of the crypt led to the perforation of the lateral plate of the alvolar process (Fig. 6). This pattern of bone apposition and resorption in the crypt indicated (a) an upward and outward migration of the tooth germ, and (b) a downward and outward growth of the cervical loop region of the tooth germ.

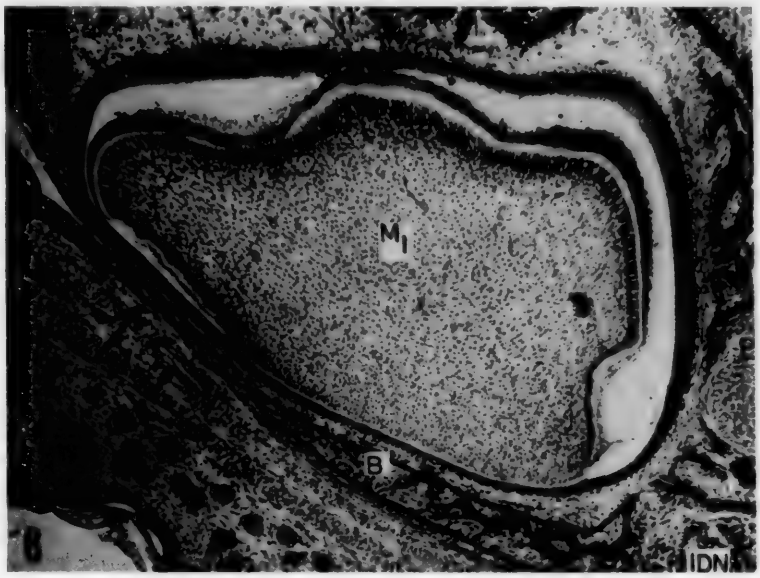


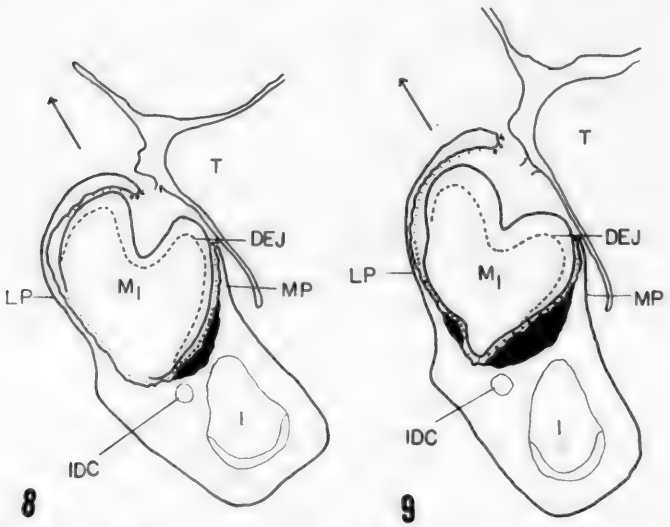
Fig. 6.—First molar tooth germ (M_1) in normal mandible at 6 days. Note complete absence of bone on the lateral (buccal) surface. Follicle is seen only on the lingual part of the tooth bud. *B*—bony plates of mandible; *IDN*—inferior dental nerve and canal. (Orig. mag. ×80.)

3. *Changes in the follicle:* At 6 and 8 days, a definite follicle could be seen only on the lingual side. At 8 days, the inter-cellular fluid decreased, the fibroblasts increased in number, and a few collagen fibers appeared, which, like the cells, were oriented parallel to the surface of the tooth germ.

C. *Ten to 20 Days After Birth.*—

1. *Tooth development:* At 10 days after birth, Hertwig's epithelial root sheath appeared and the formation of the root began. Formation of the root bifurcation, inter-radicular bony septa, and the maturation of enamel began at 12 days (Figs. 7 and 9). At 17 days after birth, the enamel, with the exception

Fig. 7.—First molar (M_1) tooth germ at 12 days after birth. *D*—dentine; *E*—enamel; *B*—bone; *RB*—root bifurcation. (Orig. mag. ×80.)



Figs. 8 and 9.—Camera lucida tracings through first molar (M_1) in normal rats at ten (Fig. 8) and 12 days (Fig. 9) after birth. Dark hatched areas indicate rapid bone formation which occurs in form of spaced bony plates. Figures show apposition (+) and resorption (−) pattern in the crypt. *Arrow* indicates the direction of tooth migration. *T*—tongue; *DEJ*—detinoenamel junction; *MP*—medial plate of bone; *LP*—lateral plate of bone; *IDC*—inferior dental canal; *I*—incisor.

of a slight cervical portion, was completely matured, and the mesial cusps of the tooth "broke through" the oral mucosa. At 19 days after birth, the remaining enamel in the cervical area matured and the distal cusps also entered the oral cavity.

2. *Movement of the tooth:* At 10 days after birth, the root formation began and the crypt showed striking changes. On the part of the crypt which faced the inferolingual surface of the crown, and where previously surface bone apposition occurred, now bone was formed in trabecular arrangement (Figs. 8 and 10). Elsewhere on the crypt wall, resorption continued.

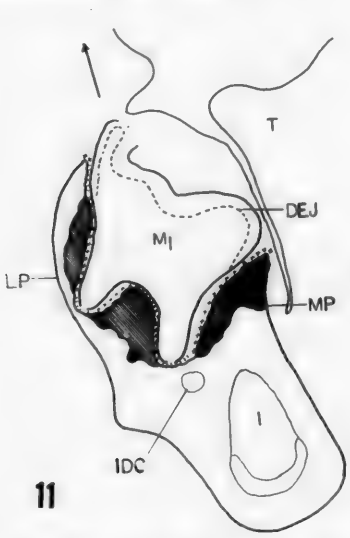
Fig. 10.—First molar tooth germ (M_1) at 15 days after birth. Bone tissue (*B*) above the black line represents newly formed trabeculae. *D*—dentin; *E*—enamel. (Orig. mag. ×80.)

At 12 days the roots of the first molar (mesial, mesiolingual, mesiobuccal, and distal) had formed to some length. At this stage, the parts of the crypt which faced the crown and the apices of the 4 roots showed inactivity or varying degrees of resorption. The crypt wall surrounding the 4 roots, however, showed varying degrees of bone apposition. Around the mesial root, trabecular bone formation occurred on the wall which faced it lingually, and surface apposition of bone occurred on the wall which faced it buccally. In the region of the mesiolingual and mesiobuccal roots, although bone apposition was seen on all walls of the crypt, it was specially rapid in the area of the bifurcation (Fig. 9). The crypt around the distal root showed similar changes as around the mesial root, except that the amount of bone apposition was less advanced.

From 12 days to about 17 days after birth, the pattern of bone apposition and resorption in the molar socket remained essentially the same. However,

rapid bone growth, as indicated by its trabecular arrangement, could now be seen on the walls of the socket facing the buccal surfaces of the mesial, mesiobuccal and distal roots (Fig. 11). As the roots lengthened and the tooth migrated occlusally, the rapid formation of trabecular bone on the walls of the socket continued. In addition, trabecular bone formation could also be seen in the areas of the bifurcation of roots. During these stages, the upper part of the socket wall facing the buccal surface of the crown consistently showed areas of resorption.

At 19 days after birth, rapid bone apposition in the molar socket ceased. At this time slow surface apposition occurred only on walls which faced the apical parts of the roots, while the upper portion of the buccal alveolar plate showed continued resorption.



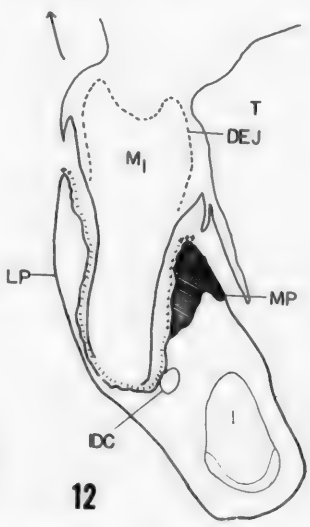


Fig. 11.—Camera lucida tracing of first molar (M_1) in the area of its mesiolingual and mesiobuccal roots. Hatched area indicates rapid bone apposition. Some parts of the socket show resorption (–). *T*—tongue; *DEJ*—dentoenamel junction; *MP*—medial plates of bone; *LP*—lateral plates of bone; *IDC*—inferior dental canal; *I*—incisor. (Orig. mag. ×18.)

Fig. 12.—Camera lucida tracing of socket around the mesial root of molar tooth at 21 days after birth. Note areas of resorption (–) and apposition (+). (Orig. mag. ×18.)

3. *Changes in the follicle:* At 12 days the tooth began rapid occlusal migration and, in the space originally occupied by it, roots began to form. From 12 to 15 days the connective tissue surrounding the crown was widest on its lingual side. The space between the roots and the surrounding bone, however, was even wider, and contained highly cellular tissue with abundant intercellular spaces and a few fibers. Between 15 and 17 days, 2 more or less distinct zones could be seen in the tissue surrounding the roots. Ovoid or cuboidal cementoblasts were seen adjacent to the root surfaces, while fusiform fibroblasts and a few fine fibers could be seen between the cementoblasts and the surrounding bone. The fibroblasts and the fibers were oriented parallel to or at slight inclination to the long axis of the root (Fig. 13).

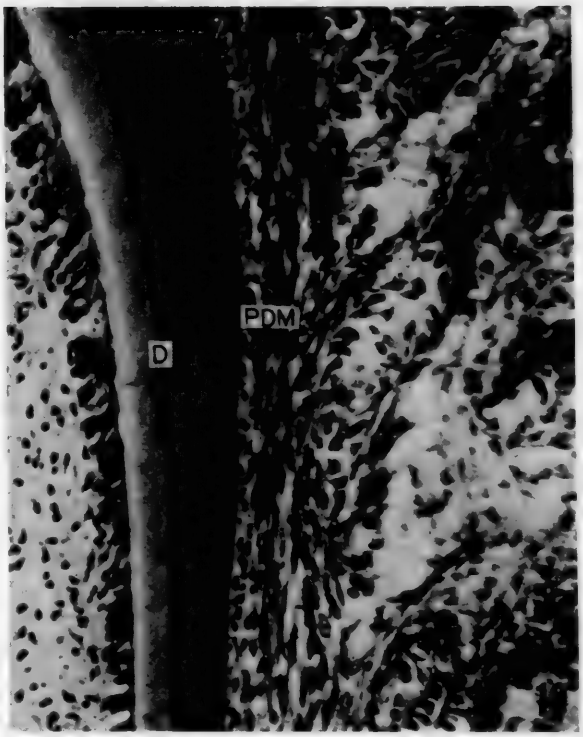


Fig. 13.—Developing periodontal membrane (*PDM*) at 15 days after birth. *D*—dentin. (Orig. mag. ×300.)

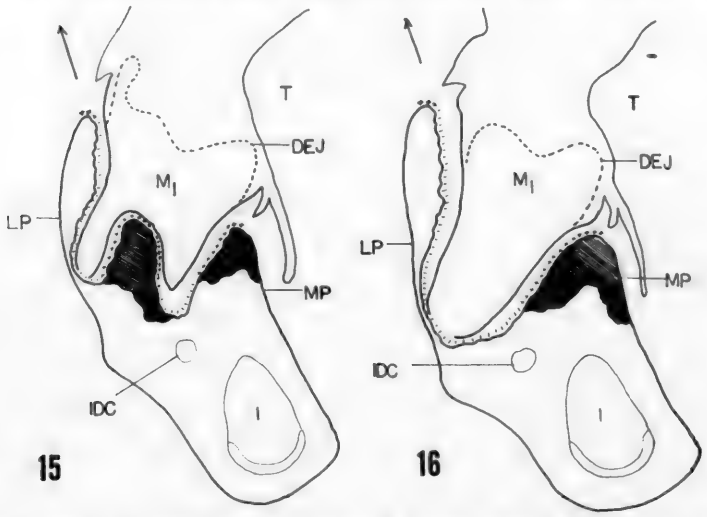
Fig. 14.—Frontal section through the mesiolingual and mesiobuccal roots at 21 days after birth. The periodontal membrane is wider on the lingual than on the buccal sides.

At 17 and 19 days, respectively, the periodontal space around the mesial and the other 3 roots began to widen (Fig. 12). This was correlated to the pattern in which the occlusal surface of the tooth erupted into the oral cavity. The periodontal space was wider on the lingual sides of the roots than on their buccal sides (Fig. 14). The general orientation of the fibers was from the region of the cementoenamel junction to the alveolar crests, and from the bone of the socket to directly across or to slightly apical areas on the root (Fig. 14). On the buccal side of the mesiolingual root, and on the lingual of the mesiobuccal root, however, the collagen fibers ran parallel to the long axis of the roots (Fig. 14).

D. *Twenty-one to 30 Days After Birth.*—

1. *Tooth development:* The first molar tooth germ came into functional occlusion at about 23 days of age. Thirty days after birth the root formation was completed and their apices began to show apposition of cellular cementum.

2. *Movements of the tooth:* At 21 days, the molar socket showed a striking change; instead of a predominance of bone apposition as seen previously, now the predominant picture was that of bone resorption. The pattern of bone apposition and resorption on the socket wall is shown in Figs. 15 and 16.



Figs. 15 and 16.—Camera lucida tracings of frontal sections through the mesiolingual and mesiobuccal roots (Fig. 15) and the distal root (Fig. 16) of first molar (M_1) at 21 days after birth. Hatched areas indicate rapid bone apposition. Apposition (+) and resorption (−) pattern of bone as shown indicates an upward and outward migration (*arrows*). (Orig. mag. ×18.)

Up to 30 days this pattern remained essentially unaltered. At 30 days, resorption in the molar socket was even more pronounced. The mesial root was surrounded by the socket wall which showed resorption throughout. In the area of the mesiobuccal and mesiolingual roots, only the crest of the interradicular septum showed surface apposition. Around the distal root, slight apposition could be seen in the upper part of the lingual wall.

3. *Changes in the follicle:* From 21 to 30 days, the periodontal membrane space continued to widen. At 23 days, the connective tissue of the periodontal membrane consisted of densely collagenized, well-oriented fibers. They extended from the bone to slightly apical areas on the root. The only exception was the buccal side of the mesiolingual root, where the fibers were oriented parallel to the long axis of the root. At 30 days, however, even in this area the fibers extended from the alveolus to the root surface.

DISCUSSION

A. *Eruptive Movement of the First Lower Molar of the Rat.*—

Weinmann[10] divided the movement of a tooth into 3 distinct phases, pre-eruptive, pre-functional, and functional. The pre-eruptive phase was designated as the movement of a tooth prior to root formation. By such movement, which occurs mainly as excentric growth, the teeth adjust their position within the jaws. The pre-functional phase begins with root formation and ends when the tooth reaches occlusion while the functional phase begins when the teeth reach occlusion and ends with tooth loss.

The present investigation further confirms the view that the movement of the tooth germ begins in the cap or bell stage and from then on continues at varying rates. From the time the first molar tooth is in cap or early bell stage, the mandible grows rapidly at its crest as well as in thickness by apposition on its lateral surface.[4] To keep up with this mandibular growth, the tooth germ moves in an upward and outward direction. If the tooth germ did not move in this manner it would soon become embedded within the jaw.

The superposition of tracings of the *ia* and normal molars shows that, up to about 5 days, the molar moves and grows in a more or less identical manner. If one superimposes tracings of *ia* molars at successive stages, it is seen that the dentinoenamel junction of this tooth progressively shifts in an upward and outward direction. Since the dentinoenamel junction is a fixed point in a tooth, its shift implies a corresponding shift of the tooth. That the tooth germ moves bodily at this early stage is illustrated further by the fact that, on the inferolingual wall of the crypt, apposition of bone or resting lines can be seen, whereas elsewhere, on the surface of the crypt, resorption is in progress. These resting lines, or bone apposition, indicate the movement of the tooth germ away from this wall. It has also been shown that on the lingual and inferolingual sides of the tooth germ the follicle is always wider than on the lateral side. This observation further indicates the bodily movement of the tooth germ.

A study of the resorption and apposition pattern of the crypt from 5 to 8 days in the normal rats indicates that the movement of the tooth remains essentially unchanged.

At 10 days, the root formation begins and the movement of the tooth becomes more rapid. From 10 to 21 days after birth, the relationship between the alveolar crest and the crown progressively changes so that the crown moves toward the oral cavity at a faster rate than the growth of the alveolar crests.

In other words, the crown progressively moves out of the jaws. During this phase, the apposition of bone is fastest on those walls of the socket which face the inferolingual and lingual surfaces of the roots. Also, during this period the newly formed periodontal membrane tends to be widest in these areas. These observations indicate that while the tooth moves axially toward the oral cavity, it also tilts in an outward direction. Since, during development, the molar crown is steeply inclined within the jaws, this outward movement of the tooth during eruption is a necessary adjustment for its normal positioning within the jaw.

From 21 days to 30 days of life, the movement of the tooth is considerably slowed. This is due to the fact that the first molar has already reached its occlusal plane.

B. *Downward Growth of the Tooth Germ.*—

Whether a tooth germ during its development grows down into the jaws has been a point of controversy.[12, 13, 24] The present investigation shows that the molar tooth germ of the rat does grow into the jaws for a short distance. A study of the *ia* first molar revealed that, up to 5 days, the molar crown is not distorted on its occlusal, lingual, or buccal surfaces. However, slight impingements by bone occurred in the area of the cervical loop, and they led to progressive distortion of the tooth. Since in *ia* jaws, resorption is absent,[2, 4] it is obvious that these cervical areas of distortion are correlated with the down growth of the cervical loop region of the tooth germ. Between 10 and 12 days, the bottom of the crypt showed beginning of bone apposition, which indicated the cessation of the down growth of the tooth germ.

C. *Correlation Between Tooth and Jaw Growth.*—

It has been shown that the molar crown is so inclined within the jaw that its occlusal plane faces inward and upward. Later when the tooth begins its movement toward the oral cavity, the crown straightens its position. The inclination of the molar crown during development is an adaptation of the tooth growth to the jaw growth. The rat has a single set of molars. The basic size of a tooth crown does not change after the establishment of the dentinoenamel junction, while the mandible of the rat grows almost continuously. The rat molar, which attains its permanent adult size very early, develops in a mandible which is comparatively much smaller. Since the transverse width of the rat molar crown in a horizontal position is much larger than in a tilted position, the development of the molars in this position makes it possible for these teeth to grow to adult dimensions in a relatively small jaw.

D. *The Follicle.*—

This study has shown further that the follicle undergoes changes which are correlated to tooth movement and function. Until the root formation begins, the follicle consists of loosely arranged stellate and fusiform cells, a few fibrils, and abundant interfibrillar substance. The width of the follicle tends to be greater on the sides from which the tooth germ is migrating than on the side to which it migrates.

As the root formation begins and the tooth moves rapidly toward the oral cavity, the connective tissue around the crown appears unchanged, but surrounding the roots there is abundant formation of new connective tissue, which is the precursor of the periodontal membrane. This tissue is at first mainly cellular, but gradually collagen fibers appear in it. In the beginning, the cells and few fibers are oriented parallel to the root surface, but as soon as the tooth reaches its occlusal plane (21 days) the fibers and cells become arranged so that they run from the bone to the cementum. Also, at this time, the bone apposition on the walls of the socket is replaced by bone resorption. This leads to a widening of the periodontal membrane. The collagen bundles of the periodontal membrane also progressively increase. These changes are correlated to the establishment of the masticatory function, and are first noted around the mesial root, then around the mesiobuccal and mesiolingual roots, and finally around the distal root. Since the eruption of the molar tooth has an anterior posterior gradient, the order in which these changes occur is not unexpected.

E. *Mechanism of Eruption.*—

The present investigation has shown that the tooth eruption begins almost simultaneously with the formation of the tooth bud. Since this is the case, this movement cannot be dependent upon the elongation of root, formation of dentin, or the apposition of enamel. Further, since the teeth erupt farther than the lengthening of their roots, one can conclude that root formation is not the cause of eruption. It was demonstrated in the present study that during some stages the eruption of the molar tooth germ was associated with inactivity or resorption on the walls of the crypt. It may be therefore assumed that the bone formation alone cannot cause eruption.

Essentially, eruption is the movement of one organ (tooth) in relation to the other (the bony crypt or socket). This phenomenon is not unique to the teeth but is commonplace in ontogenesis. The "movement" of the heart from the visceral arch region to the thorax, of the thyroid anlage from the base of the tongue to the neck, of the eyes from the lateral to the anterior aspects of the face are all examples of organ migration or movement. These shifts in organ position are undoubtedly a result of differential growth and directional growth. Eruption, like other organ movements, appears to be the result of different rates of growth of the dental pulp, dental follicle, and the bony crypt. To simplify the mechanism of tooth eruption, one may compare it with the growth or "movement" of 2 bones as a result of sutural growth. The growth of connective tissue of a suture leads to a "pushing apart" of the participating bones. Following this, bone apposition occurs on the opposing surfaces so that the suture is maintained at a more or less constant width.

From the present investigation it would appear that the eruption of teeth is probably similar to the sutural growth and the connective tissue of the dental follicle and periodontal membrane is analogous to the connective tissue of the suture. The growth of the follicular connective tissue leads to the migration of the tooth germ or tooth (comparable to one of the bones) from the bottom

of the crypt (comparable to the second bone) (Fig. 17, *A* and *B*.) This process is simultaneously associated with the lengthening of the tooth as well as by the apposition of bone on the walls of the crypt (Fig. 17, *C*). These changes are therefore analogous to the growth of the opposing bones. It is thus obvious that root lengthening and apposition of bone in the socket are not the cause but, rather, are the result of tooth eruption. The migration of teeth or tooth germs while the crypt shows bone resorption and their movement prior to the formation of the root support this view. That the connective tissue growth is the primary factor in eruption is further supported by the observation that, when connective tissue proliferation is suppressed, tooth eruption is retarded.[27, 28]

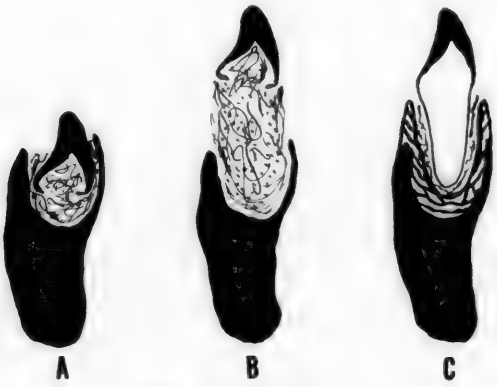


Fig. 17.—*A*, *B*, and *C*.

SUMMARY

This histologic study on the eruptive movement of the rat first molar is based on serial frontal sections through the lower first molar region of 20 *ia* and 34 normal rats ranging in age from 13 days' insemination age to 30 days after birth. Since the *ia* rats are characterized by absence of bone resorption, a number of fixed points are retained in the *ia* mandible. These fixed points permit accurate comparisons of succeeding developmental stages in the *ia* animals. A comparison of *ia* and normal rats was made to study the areas and role of bone resorption during tooth movements. The findings follow.

1. The developing tooth of the rat first molar is initiated at 13 days' insemination age. Between 20 and 21 days' insemination age, the inner enamel epithelium differentiates into ameloblasts, and adjacent connective tissue cells form odontoblasts, with subsequent dentin formation at 21 days' insemination age. At 3 days after birth, enamel formation begins. At 10 days after birth, the formation of the root begins, and Hertwig's epithelial sheath appears. The tooth "breaks through" the oral epithelium around 17 days after birth and comes into functional occlusion at 23 days. Roots are completed at 30 days after birth.

2. The movement of the tooth germ (eruption) begins soon after its initiation and continues until 30 days of age. Prior to the beginning of root formation, the tooth germ moves upward and outward and thus keeps a constant relation with the growing alveolar process. Associated with the beginning of root formation, the tooth moves more rapidly toward the oral cavity and tilts outward. This movement is accompanied by selective, rapid bone formation on crypt wall. After the tooth comes into functional occlusion the apposition of bone in the crypt ceases.

3. The cervical loop region of the tooth germ grows deeper into the jaws for a short distance.

4. During development the crown of the first molar is inclined in the jaws in such a way that its occlusal surface faces upward and inward. This tilted position of the tooth is corrected during eruption. The tilted position of the developing crown is due to the fact that the molar teeth of "adult size" develop in a relatively smaller jaw.

5. In early stages of development, the dental follicle consists of loosely arranged mesenchymal cells with abundant intercellular fluid. With age the follicle changes to a highly cellular structure with fusiform cells arranged parallel to the tooth surface. During these stages, the follicle is wider on the lingual and inferolingual sides of the tooth germ. These are the sites from which the tooth germ migrates. Still later, collagen fibers appear among the fusiform cells. When the tooth erupts into the oral cavity, further organization of periodontal fibers occur, and they extend from the bone to the cementum. When the tooth comes into functional occlusion, the periodontal membrane becomes densely collagenized and the periodontal space widens. The widening is a result of resorption on the socket wall.

6. The eruption is not a result of root elongation, bone apposition, dentin or enamel formation, but probably is a result of growth of the follicle. This growth is analogous to the growth in a suture. The connective tissue growth in the follicle leads to migration of tooth from the crypt. It is suggested that as in the bones at a suture, the growth of the root and apposition at the bottom of the crypt are the results of tooth migration and not its cause.

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HORMONAL CONTROL OF THE PROTEOLYTIC ACTIVITY OF THE SUBMAXILLARY GLAND AND THE PANCREAS

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LACASSAGNE'S description of a sex difference in the submaxillary glands of mice has been confirmed by many studies.[1, 3, 5, 7] It has been shown that this difference can be abolished by castration and enhanced by androgens.[2, 8, 10] In the rat, there is no difference in the morphology of the glands of males and females, but there is a difference in the levels of proteolytic activity of gland extracts.[11] It was found that this difference in enzymatic activity developed after the onset of puberty and that the reduction in proteolytic activity which followed hypophysectomy could be reversed by the administration of testosterone and thyroxine.[11, 12] From these findings it was concluded that the proteolytic activity in the submaxillary gland of the rat was under control of the male sex hormone as is the morphologic sex differentiation in mice.

Since similar studies on the proteolytic enzyme systems of other glands have not been made, it was not possible to say whether this hormonal dependence was characteristic for the submaxillary gland alone. In the attempt to compare the submaxillary enzyme system with the tryptic activity of the pancreas, it was found that nonactivated extracts of rat pancreas have only a low degree of proteolytic activity. However, no standard procedure for the activation of crude pancreatic extracts was found in the literature. In this report, a procedure for standardizing the activation of pancreatic extracts by duodenal extracts and for obtaining maximal proteolytic activity in activated pancreatic extracts is presented. A comparison of the postnatal changes in the proteolytic enzyme systems of the pancreas and submaxillary gland, and the effects of castration on the 2 enzyme systems is presented.

ACTIVATION OF CRUDE PANCREATIC EXTRACTS

Adult male rats (125 to 135 days of age) of the Hunt-Hoppert caries-susceptible strain[4] were used. The animals were killed with ether and the pancreas was dissected out, placed in liquid nitrogen, and then transferred to a freeze-drying unit† for lyophilization.

An extract was made by grinding frozen-dried pancreas tissue in a mortar and adding saline solution to a final concentration of 1 mg. per cubic centimeter. This material was centrifuged for 5 min. at 1,750 r. p. m., and the supernatant fluid was used for assay.

This investigation was carried out under Contract DA-49-007 MD-622 with the Medical Research and Development Board, Office of the Surgeon General of the United States Army.

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†Wilkins-Anderson Co.

Viodenum powder* was used as the starting material. This preparation is stated to be "raw duodenum substance, desiccated and defatted at 37° C." Preliminary extracts, prepared according to Lin and Ivy[9] were obtained by shaking a 1 per cent concentration of Viodenum in phosphate buffer of pH 6.5 for 15 min. The resulting suspension was centrifuged at 3,000 r. p. m. and the supernatant fluid used as the preliminary activating agent.

One milliliter of pancreatic extract was briefly shaken with samples of the Viodenum extracts corresponding to 10 mg. of initial dry weight. The mixture was adjusted to the desired set of conditions and allowed to stand for the desired time interval. At the end of this period, 1 c.c. of substrate (1 per cent casein) was added, the pH adjusted to 6.5 and enzyme digestion allowed to proceed for 30 min. at 37° C. Protein breakdown was determined according to the method of Kunitz and the results expressed as the optic density per milligram of nitrogen.[6] The resultant optic densities were used as the measure of the activating potencies of the duodenal extracts.

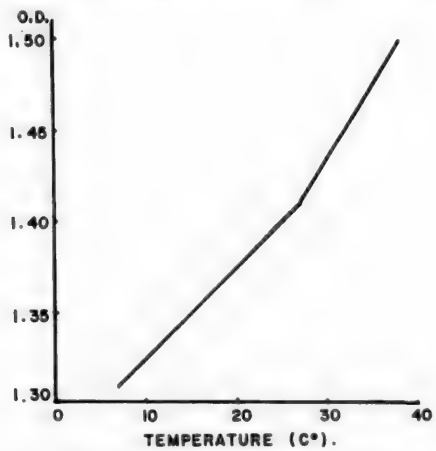

Fig. 1.—Effect of temperature on activation of pancreatic extracts by Viodenum.

The activating potency per unit dry weight of Viodenum powder was found to remain the same when using saline solution or phosphate buffer of pH 6.2 as the extracting medium, or when changing the concentration of Viodenum within a range of 1 to 4 per cent of initial dry weight.

Findings.—

A. *Conditions for maximal activation of pancreatic extracts:*

1. Optimal temperature of exposure.— With activation times of 2.5 hr., the degree of activation was higher at higher temperatures, but not very markedly so. The activating potency increased less rapidly in the temperature interval 7° to 27° C. than in the interval 27° to 37° C. (Fig. 1).

2. Optimal duration of activation.—The degree of activation rose up to about 6 hr. exposure time (Fig. 2). Then it declined slowly, but remained high

*Supplied by the Viobin Corp.

for the next 10 hr. The rise of potency was steepest between 1.0 and 3.0 hr. exposure time. In 2.5 hr. of exposure, the proteolytic activity of pancreatic extracts was raised about 45 fold over that of nonactivated samples.

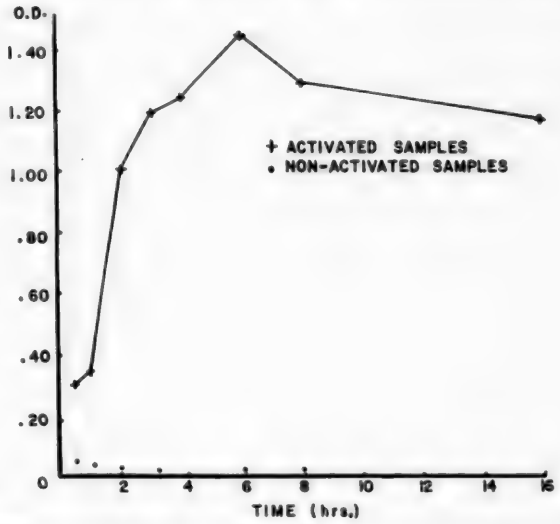

Fig. 2.—Effect of exposure time on the activation of pancreatic extracts by Viodenum.

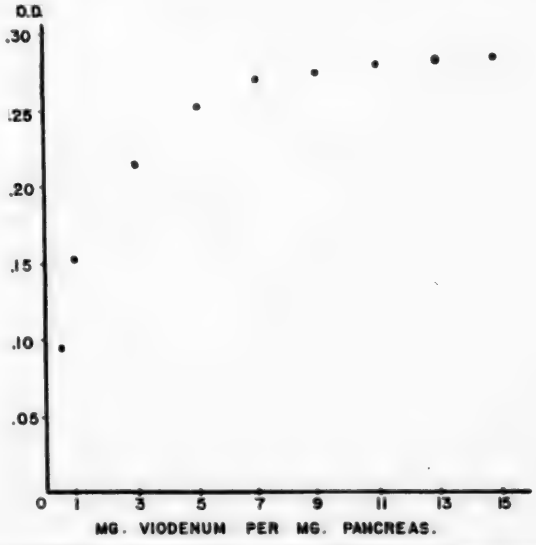

Fig. 3.—Effect of Viodenum concentration on the activation of pancreatic extracts.

3. Optimal ratio of duodenal to pancreatic extracts.—The degree of activation increased as a nonlinear function of the ratio of Viodenum to pancreas and tended to an asymptotic maximum (Fig. 3). The increase in activation was slight with ratios higher than 10 for the dry weights of Viodenum and pancreas.

4. Optimal pH for activation.—The final pH of the activating mixture was varied from 6.2 (saline) to 9.2 (borate buffer). Stable degrees of activation were obtained up to pH 8.7. Activity fell off sharply at pH values higher than 8.7.

B. *Conditions for obtaining maximal proteolytic activity in activated pancreatic extracts:**

1. pH for maximal proteolytic activity.—The proteolytic activity was found to fall rapidly below pH 6.2 and above 10.5. Changes in pH between 6.5 and 10 had only a slight effect (Fig. 4). The various buffers used (acetate, phosphate, maleate, and borate) did not affect the level of activity.

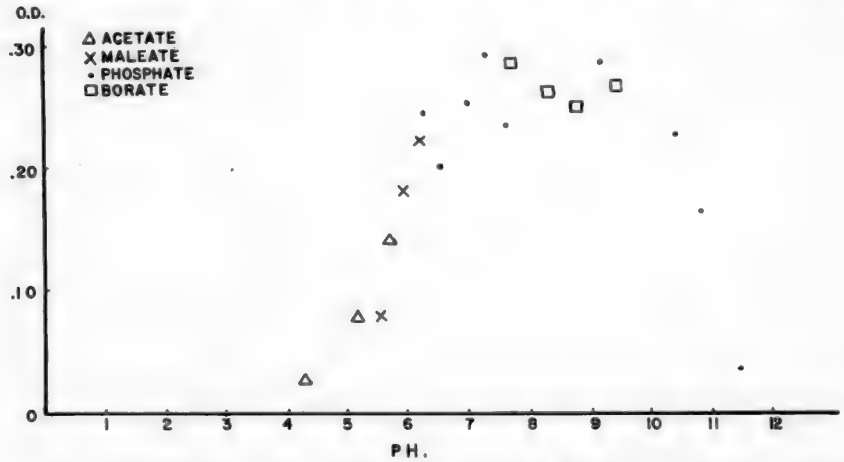

Fig. 4.—Effect of pH on the proteolytic activity of activated pancreatic extracts.

2. Wave length of maximal light absorption.—At pH 6.5, the amount of light absorbed by the proteolytic breakdown products was found to be maximal at 275 mμ. Changes in pH affected the amount of light absorbed at all wave lengths, but did not change the location of the maximum (Table I).

TABLE I
LIGHT ABSORPTION IN ACTIVATED PANCREATIC EXTRACTS

Wave length (mμ)	270	275	280
pH of Final Mixture		Optic Density	
6.19	0.183	0.205	0.196
6.54	0.269	0.282	0.259
6.99	0.208	0.233	0.208
7.58	0.219	0.242	0.229

3. Effect of varying the amount of pancreatic tissue.—The amount of pancreatic tissue was varied, retaining a ratio of 10 for initial dry weight of Viodenum and pancreas, 1 c.c. of 1 per cent casein per cubic centimeter of

*All samples of pancreatic extracts were activated using the following procedure: (1) extraction of Viodenum powder in saline at pH 6.2 at 1 per cent concentration, (2) temperature, 37° C., (3) time of exposure, 2.5 hr., and (4) initial dry weights of Viodenum and pancreas, 10:1.

pancreatic extract, and pH 6.5. Proteolytic breakdown was proportional to the amount of pancreatic tissue between 0.25 and 1.25 mg. dry weight per cubic centimeter of saline, then fell off (Fig. 5).

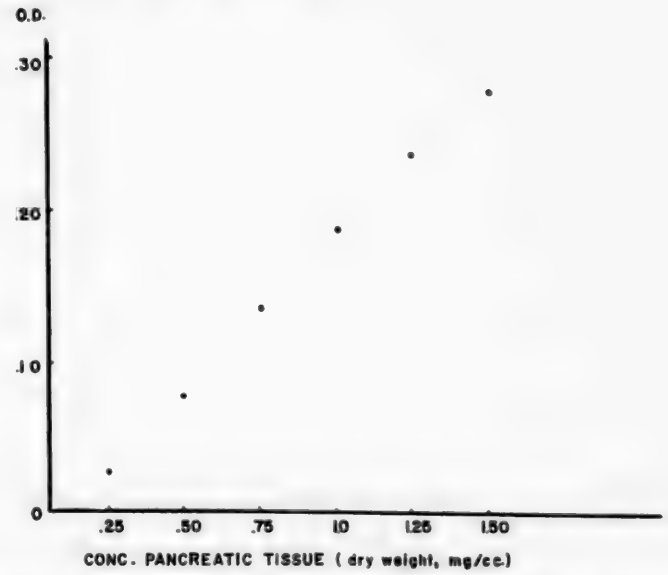

Fig. 5.—Effect of concentration of pancreatic extract on the proteolytic activity of activated pancreatic extracts.

COMPARISON OF PANCREAS AND SUBMAXILLARY GLAND

Protease activity was determined according to the method of Kunitz.[6] Saturating substrate conditions and optimal pH for pancreas and submaxillary gland were determined for the group with the highest activity (150-day-old animals) and kept standard for all other groups, as follows:

	Submaxillary Gland	*Pancreas*
Substrate	1% Casein	1% Casein
Concentration of gland extract (dry weight)	5 mg./c.c.	1 mg./c.c.
Incubation time	30 min.	30 min.
pH	9.6	6.4
Temperature (° C.)	37	37

Findings.—

A. *Postnatal changes in specific proteolytic activity:* Table II shows the changes in proteolytic activity per milligram of nitrogen which occur in the pancreas and submaxillary gland in rats between 60 and 150 days of age.

Activated samples of the pancreatic extract were 100 times as active as extracts of the submaxillary gland at 60 days of age. Subsequently, however, the specific proteolytic activity of the submaxillary gland rose about fivefold, the rise being particularly steep during the immediate postpuberal development. The activity of the pancreas remained unchanged.

TABLE II

SPECIFIC PROTEOLYTIC ACTIVITY (EXPRESSED AS OPTIC DENSITY AT 275 mμ) OF SUBMAXILLARY GLAND AND PANCREAS IN RATS OF DIFFERENT AGES

AGE IN DAYS		OPTIC DENSITY	
		PANCREAS	SUBMAXILLARY GLAND
60	Average	13.0	0.13
	Range	12.3-13.9	0.07-0.22
	No. of animals	3	3
90	Average	13.0	0.45
	Range	11.4-15.0	0.23-0.52
	No. of animals	7	7
150	Average	11.8	0.56
	Range	10.7-12.9	0.36-0.65
	No. of animals	5	5

B. *Effect of castration:* Animals used in studying the effects of castration were castrated at 30 days of age and allowed to live for between 30 to 120 days following the operation.

The effect of castration on the specific proteolytic activity of the 2 glands is shown in Table III.

TABLE III

EFFECT OF CASTRATION ON THE SPECIFIC PROTEOLYTIC ACTIVITY (EXPRESSED AS OPTIC DENSITY AT 275 mμ) OF SUBMAXILLARY GLAND AND PANCREAS IN RATS OF DIFFERENT AGES

AGE IN DAYS		PANCREAS		SUBMAXILLARY GLAND	
		NORMAL	CASTRATE	NORMAL	CASTRATE
60	Average	13.0	10.23	0.13	0.11
	Range	12.3-13.9	6.2 -12.5	0.07-0.22	0.09-0.14
	No. of animals	3	4	3	3
90	Average	13.0	13.37	0.45	0.16
	Range	11.4-15.0	12.3 -14.6	0.23-0.52	0.09-0.26
	No. of animals	7	7	7	7
150	Average	11.8	10.99	0.56	0.30
	Range	10.7-12.9	9.6 -12.2	0.36-0.65	0.22-0.40
	No. of animals	5	7	5	5

Castration had no effect on the proteolytic activity of the pancreas. The submaxillary gland was probably not affected at 60 days of age. At 90, however, activity was cut down to one third of normal values and to one half at 150 days of age.

SUMMARY AND CONCLUSIONS

Male albino rats of the Hunt-Hoppert caries-susceptible strain were used for establishing a procedure for the activation of the proteolytic activity of crude pancreatic extract by duodenal extracts; for determining optimal conditions for assaying the proteolytic activity of activated pancreatic extracts; for studying the postnatal changes in proteolytic activity of pancreas and submaxillary gland, and for studying the effects of castration on the proteolytic activity of the pancreas and submaxillary gland.

Activation of crude pancreatic extracts raised the proteolytic activity about 45 fold. At 60 days of age, the activity of pancreatic extracts per milligram of nitrogen was about 100 times as high as that of submaxillary gland extracts. No increase occurred with age, whereas specific activity of the submaxillary increased rapidly between 60 and 90 days of age. Castration did not affect the level of pancreatic activity, but reduced that of the submaxillary gland to about a third of the control values.

It is concluded that the dependence of the proteolytic activity on the male sex hormone is characteristic for the submaxillary gland and not the pancreas.

The authors wish to express their appreciation to Dr. C. A. Hoppert, Dr. H. R. Hunt, and Dr. S. Rosen at Michigan State University for their cooperation in furnishing and maintaining the animals used in this study.

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MASTICATORY APPARATUS OF ALBINO RATS IN EXPERIMENTAL LATHYRISM

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EXPERIMENTAL lathyrism, produced by feeding diets containing substantial amounts of sweet pea *(Lathyrus odoratus)* seeds, involves lesions in the skeletal system and other mesenchymal tissues. The purpose of the present investigation was to study the manifestations of experimental lathyrism in the dental and supporting structures of rat molars and incisors and in the mandibular articulation. A thorough search of the literature revealed no report on the histologic changes occurring in the masticatory apparatus of the rat or any other animals, following experimental lathyrism.

The term lathyrism has been used to refer to at least 2 different syndromes arising as the result of the ingestion of certain legumes belonging to the Lathyrus family. The early literature on human lathyrism has been comprehensively reviewed by Schuchardt.[24] Stockman[29] has perhaps given the best description of its symptomatology. The species of Lathyrus responsible for lathyrism in man *(L. sativus, L. cicera,* and *L. clymenum)* are also toxic to certain other animal species,[1, 2, 28, 29] but are nontoxic to rats.[14] The symptoms of this type of lathyrism are usually attributed to central nervous system damage, and damage to the brain and spinal cord has been demonstrated in experimental animals.[29a] Selye[25a] has recently introduced the term "neurolathyrism" for this syndrome.

A second type of lathyrism can be produced in rats by feeding seeds of *Lathyrus odoratus* (sweet pea),[9, 14] *L. hirsutus* (Caley pea),[14] *L. pusillus* (Singletary pea),[11a] or *L. tingitanus,*[14] or by feeding certain amino-nitriles,[3a, 8a, 35] notably beta-aminopropionitrile. Because of the spectacular skeletal changes which are produced, this type of lathyrism has been termed "osteolathyrism."[25a] It is known to occur also in cattle[31] and horses[32] and has been produced in poultry,[3b, 3c] chicken embryos,[5] and amphibians.[5, 13] Schilling and Strong[21, 22, 23] identified the crystalline toxic compound isolated from *L. odoratus* as beta (gamma-l-glutamyl) aminopropionitrile.

Clinical Symptoms.—The experimental feeding of sweet peas or beta-aminopropionitrile produces a decrease in the rate of growth[8, 9, 15] and marked skeletal changes. The latter were characterized by kyphoscoliosis in the thoracic

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region,[9, 12, 14, 15, 17, 19, 30, 34] sternal curvature,[9, 35] dislocation of joints,[5, 16] deformation of humerus and femur,[5, 7a, 9] and hemarthrosis.[14, 15] Dissecting aortic aneurysms occurred and often resulted in ruptured aortas.[3, 11, 19, 27] Pyörälä, Seppala, and Punsar[19] and others[27, 37, 38] found that when sweet pea seed was fed toward the end of gestation, aortic and skeletal changes occurred in the young.

Histologic Changes.—The histopathologic changes observed by various investigators are related primarily to changes in mesenchymal tissues and include the following: degeneration of intervertebral discs,[15, 16] enlargement, widening, and disruption of epiphyses of long bones,[12, 14, 16, 18, 35, 36] osteoporosis,[9, 10, 14, 15, 21, 25, 34] disuse atrophy in bones,[10] periosteal new bone formation at the sites of muscle attachments,[9, 14, 16, 25, 35, 39] loosening and detachment of tendinous and ligamentous insertions,[5, 16] degenerative arthritis,[16, 36] proliferation of connective tissue into the neighboring muscle,[18] replacement of joint cartilage by connective tissue,[16, 18] tearing of the periosteum,[10, 16, 18] new formation of cartilage,[17] dissecting aortic aneurysm, edema, and medial hemorrhage,[3, 4, 6, 11, 15, 16, 17, 19, 35] and fragmentation and lysis of elastic fibers of the aortic media.[3, 6, 19, 27, 36] Degenerative changes observed in the spinal cord[16] were explained by primary changes in the angulation of the spine. Levy and Goodman[13] observed the development of tumors of the notochord of *Amblystoma punctatum* larvae due to changes in the connective tissue sheath surrounding the notochord.

Changes in the Jaws and Teeth of Animals.—Ungar and Sciaky[33] were the first to demonstrate in anatomic and radiographic specimens an abnormal inclination of the lower molars and morphologic alterations in the periodontal membrane. Dasler[7a] observed exostoses on the ventral and lateral aspects of the mandible following the feeding of a 50 per cent sweet pea diet to weanling rats. The exostoses were palpable in all rats at the end of 1 week, and became progressively larger with the duration of the experimental feeding. He further called attention to a notching of the incisors in growing rats that were fed sweet peas. He suggested that this notching was due to the overgrowth of bone in the proximal portions of the mandible, which limited the range of movement of the jaws and thus interfered with the normal attrition of the incisor.

Dasler[7] and Geiger[9] found a decrease in the ash content of the bones of young experimental rats while the incisors[7] showed a slight increase.

Levy and Goodman[13] reported deformities of the mandible of *Amblystoma punctatum* embryos that were reared in an aqueous solution of crystalline Lathyrus factor.

MATERIALS AND METHODS

This investigation is based on a macroscopic and microsopic study of the jaws and dentition of 14 experimental rats and of 14 control littermates ranging from 22 to 43 days of age. Weanling albino rats of the Holtzmann-Sprague-Dawley strain, 22 days of age and weighing 41 to 52 Gm., were fed *Lathyrus odoratus* (sweet pea) diets.

The sweet pea diets had the following composition: pea meal 50 per cent, casein 10 per cent, cornstarch 25 per cent, sucrose 5 per cent, brewer's yeast powder 4 per cent, and cod liver oil 2 per cent. The dry ingredients were mixed separately and stored, the cod liver oil being added to portions of the mixture as needed. The pea meal was prepared by grinding to a fineness which permitted all the material to pass through a 24-mesh sieve. Control littermates of the same weight were pair-fed the same basic diet, but the sweet pea meal was replaced by the same proportion of edible pea meal. The feedings were continued for 1- 2- and 3-week periods, respectively.

After the animals were killed, the skulls were divided into halves, fixed in 10 per cent formalin and radiographed. With a millimeter caliper the following measurements were taken from each radiograph (Figs. 1, *A* and *B*). (1) The length of the skull, from the nasion to the external occipital crest of the occipital bone. (2) Upper diastema, i.e., the space between the lingual alveolar crest of the upper incisor and the mesial surface of the upper first molar at the level of the mesial alveolar crest. (3) Lower diastema, i.e., the distance from the lingual alveolar crest of the lower incisor to the mesial surface of the lower first molar at the level of the mesial alveolar crest. (4) The distance between the occlusal surface of the first lower molar and the superior surface of the lingual alveolar bone of the lower incisor. (5) The extra-alveolar length of the lower incisor. (6) The extra-alveolar length of the upper incisor, and (7) the length of the mandible, from the notch between the angular and condyloid process to the lingual alveolar crest of the symphysis.

In addition, a representative group of skeletal specimens of the cranium and mandible were macerated and used for gross study.

After sacrifice, the heads were decalcified in 5 per cent nitric acid. The upper and lower jaws were dissected and the upper molars, upper incisors, and mandibular articulation were embedded separately in celloidin or paraffin. Mesiodistal sections through two buccal roots of the upper molars, sagittal sections of the incisors, and frontal sections of the mandibular articulation were cut and stained with hematoxylin and eosin and Mallory connective tissue stain, or impregnated with silver according to the method of Gomori.

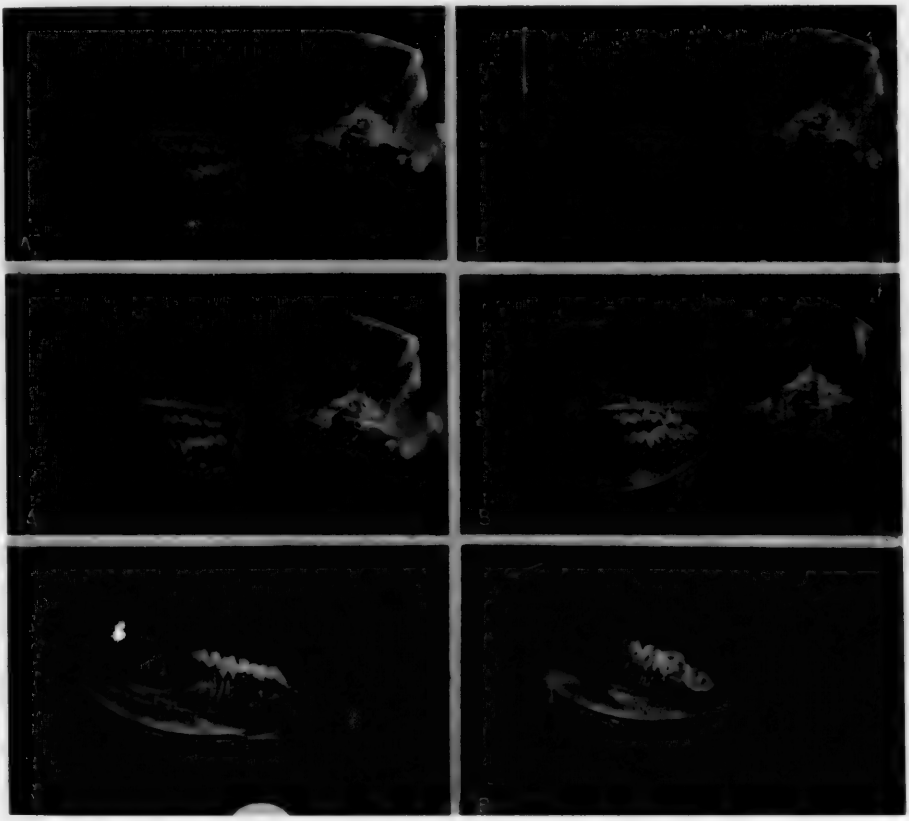
The width of the periodontal membrane, dentin, and cementum was measured from central sections of the upper second molar in control and experimental animals. The distance between the highest point of the alveolar crest and the cemento-enamel junction was measured at the mesial and distal surface of the upper second molars. Measurements were also taken of the width of the periodontal membrane, labial alveolar periosteum, dentin, cementum, enamel matrix, and fundic connective tissue of the upper incisors of control and experimental animals.

FINDINGS

Gross Changes.—

Exostoses on the ventral and lateral aspects of the mandibles could be palpated within 1 week. Marked spinal curvatures or thoracic cage deformities were evident in about 2 weeks.

The food consumption increased more or less in proportion to the gain in weight for about the first 12 days of feeding. In 1 rat in the 3-week group, the food consumption was maintained at the maximum level for the remainder of the 21-day period. In the remaining rats, food consumption fell off in varying amounts, beginning at about the nineteenth day of the feeding. One of the experimental rats in the 3-week group died on the nineteenth day, another died



Figs. 1*A* and 1*B*.—Radiographs of skull of control animal, 43 days of age, showing location of landmarks from which measurements were made of both control and experimental animals.

Figs. 2*A* and 2*B*.—Radiographs of the skulls of control (*A*) and experimental (*B*) animals, 43 days of age, fed their respective diets for 3 weeks. In *B* note exostoses on coronoid (*1*), and angular processes (*2*), the ventral surfaces of mandible (*3*), at external occipital crest (*4*).

Figs. 3*A* and 3*B*.—Radiographs of mandible of control (*A*) and experimental (*B*) animals, 43 days of age, after 3 weeks of their respective diets. Note deformation of condylar process (*1*), exostoses on the coronoid (*2*) and angular processes (*3*), the ventral surface (*4*), abnormal lingual positioning of molars (*5*), and notch at the incisal surface (*6*).

on the twenty-first day. The latter had paralysis of the rear legs beginning at about the eighteenth day. A decrease in activity and muscle tone became evident in varying degrees in most rats after more than 2 weeks of feeding.

The control groups gained weight in excess of the experimental animals. The experimental rats showed a 43 per cent increase in body weight in the first

experimental week compared to an increase of 56 per cent in the controls. In the first 2 experimental weeks the experimental rats increased 106 per cent in body weight compared to 122 per cent in the controls. The percentage increase in weight in 3 weeks of experimental feeding was 140 per cent in the experimental animals compared to 219 per cent in the controls.

Studies of anatomic specimens and of radiographs of craniums and mandibles showed in the experimental animals: (a) a lower rate of growth of the skull and slower eruption of the teeth, (b) development of exostoses, (c) changes in the mandibular articulation, and (d) changes in the pattern of attrition.

The 7 sets of measurements taken from radiographs of control and experimental animals were so selected that they could be taken from accurate landmarks, in regions which showed demonstrable growth in the control animals during the period covered by the experiment. At the end of the experimental period all 7 measurements in the experimental animals were smaller than in the controls. The differences in the absolute size reached ranged from 5 per cent for the lower diastema to 38 per cent for the extra-alveolar portion of the upper incisors (Table I). The degree to which growth was inhibited in the experimental animals varied from complete failure to gain in length (upper incisor) to almost the same rate of growth (lower diastema) as in the controls.

TABLE I

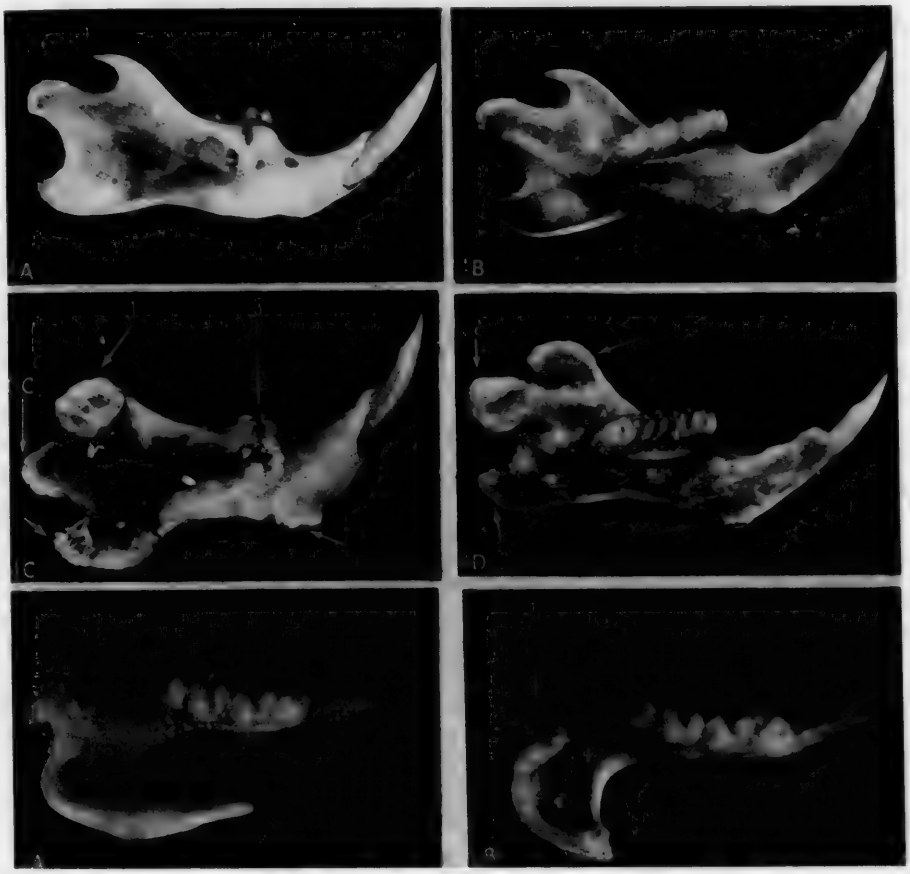
PERCENTAGE DIFFERENCES BETWEEN THE ROENTGENOGRAPHIC MEASUREMENTS OF EXPERIMENTAL ANIMALS AND THEIR PAIR-FED CONTROLS AFTER 3 WEEKS ON THE EXPERIMENTAL DIET

SITE MEASURED	DIFFERENCE IN SIZE OF EXPERIMENTAL ANIMALS AFTER 3 WK. EXPRESSED IN PERCENTAGE OF THE CONTROL MEASUREMENTS (%)
1. Cranium	–7
2. Upper diastema	–9
3. Lower diastema	–5
4. Distance between occlusal of first lower molar and superior surface of lingual alveolar bone	–10
5. Extra-alveolar portion of lower incisor	–20
6. Extra-alveolar portion of upper incisor	–38
7. Mandible	–11

Exostoses of different shape and size developed in the cranium and mandible in the areas of muscle attachments. In radiographs the exostoses appeared as irregular circumscribed radiopaque areas (Figs. 2, 3, 4, and 5). The surfaces of the mandible that were free of muscle attachments were smooth.

The following areas of muscle attachments on the mandible, maxilla, and occipital bone showed exostoses: (1) The angular process where the internal pterygoid muscle and a part of the masseter muscle are attached; (2) the masseteric ridge and a field above its anterior end below the first molar, the area of attachment of one portion of the masseter muscle; (3) the coronoid process, the area of attachment of the temporal muscle; (4) the medial aspect of the mandibular neck, the area of insertion of the external pterygoid muscle;

(5) the anterior part of the lower border of the mandible close to the symphysis, the area of attachment of the digastric and transverse mandibular muscles; (6) the body of the maxilla close to the premaxillary process where the anterior superficial part of the masseter muscle inserts; and (7) the external occipital crest of the occipital bone where muscles of the neck are attached.



Figs. 4*A*, 4*B*, 4*C*, 4*D*.—Photographs of the mandible of control (*A*, *B*) and experimental (*C*, *D*) animals, 43 days of age, after 3 weeks on their respective diets. *A*, *C*, lateral views; *B*, *D*, medial views. Note exostoses on the coronoid (*1*), angular processes (*2*), lateral (*3*), ventral (*4*) surfaces of the mandible of experimental animals and, *cc*—condyle.

Figs. 5*A* and 5*B*.—Photographs of the maxilla of control (*A*) and experimental (*B*) animals, 43 days of age, which were fed their respective diets for 3 weeks. In *B*, note exostoses anteriorly to the first molar at the site of attachment of the anterior superficial part of the masseter muscle (*1*).

Changes in the mandibular articulation were mainly restricted to the condylar process of the mandible. The condyle itself was enlarged in an anteroposterior direction. The normally smooth articular surface was rough and irregular, as was the mandibular neck due to an exostosis on its medial surface, where the external pterygoid muscle inserts.

The lower incisors were notched at their incisal surface in constrast to the sharp incisal bevel in the controls. This was apparently an expression of a change in the pattern of attrition of the incisor teeth. The upper incisors were notched in a few instances but not to as marked a degree as were the lower incisors (Fig. 3, *B*). The upper molars and the first lower molars maintained a normal position. The lower second and third molars showed an abnormal lingual inclination (Fig. 3, *B*).

Microscopic Findings.—

Molars: The following description is based primarily on a study of the second molar because this is the only tooth from which central sections permitting quantitative comparisons were available. The qualitative changes in the first upper molars were similar to those in the second molars. The third molar, which erupts at approximately 40 days, could be used only for limited aspects of the study. The most marked changes in the molars and their supporting tissues were seen in the periodontal membrane.

The enamel organ of the unerupted upper third molar showed edema of the cells in the papillary and ameloblastic zones. The cytoplasm of these cells contained vacuoles and pyknotic nuclei.

The dentin showed a narrow layer of disturbed calcification, a calciotraumatic line, which separated the pre-experimental from post-experimental dentin. As early as after 1 week of feeding, the newly formed dentin in the root, especially in the area of the apex, showed a disturbance of calcification in the form of interglobular dentin. The amount of the interglobular dentin increased with the duration of the experiment. The dentin, in the 3 areas measured, increased in thickness with the duration of the experimental period. However, the rate of growth of the dentin in the experimental animals ranged only from 18 to 55 per cent of that of the controls (Table II) after 3 weeks.

The pulps of animals fed with the sweet pea diet for 2 and 3 weeks showed dilated capillaries associated with small hemorrhages; otherwise no significant changes in the pulpal connective tissue could be observed.

The roots of the upper second molars were straight and conical after 1 week of the experimental feeding, but showed a progressive bending at the apical region after the second and third weeks. Such deformities were not observed in the control animals. In the experimental animals, they were apparently caused by an interference with eruption.

Immediately below the cemento-enamel junction a spur of cementum developed in the second and third weeks of experimental feeding, which was not observed in the control animals. The spur was stained pink by eosin and resembled cementoid tissue. The acellular cementum increased in thickness in both control and experimental animals. The increase was slightly smaller in the experimental animals. The thickness of the cellular cementum of control animals was greater at the mesial surface than at the distal surface of the roots. However, in the experimental animals it was greater at the distal surface of the roots. The rate of growth of the cellular cementum in the experimental animals was demonstrably slower. It ranged from 32 per cent of that of the controls

to only 9 per cent of the controls in the different areas measured (Table II) after 3 weeks. This was demonstrated to be statistically significant at the 1 per cent level of confidence at all 4 sites measured.

TABLE II

PERCENTAGE DIFFERENCE IN GROWTH OF CEMENTUM AND DENTIN OF UPPER SECOND MOLAR IN EXPERIMENTAL AND CONTROL ANIMALS AFTER 2 AND 3 WEEKS ON THE EXPERIMENTAL DIET, AS MEASURED ON HISTOLOGIC SECTIONS

	EXPERIMENTAL AND CONTROL INCREMENTS OF GROWTH (μ)		PER CENT OF CONTROL GROWTH RETAINED	
	AFTER 2 WK.	AFTER 3 WK.	AFTER 2 WK.	AFTER 3 WK.
Tissue measured and site of measurement	$\frac{\text{exp. } x_2\text{-}x_1}{\text{cont. } x_2\text{-}x_1}$	$\frac{\text{exp. } x_3\text{-}x_1}{\text{cont. } x_3\text{-}x_1}$	$100\ \frac{x_2\text{-}x_1}{x_2\text{-}x_1}$	$100\ \frac{x_3\text{-}x_1}{x_3\text{-}x_1}$
Dentin-Molar distal root	$\frac{181.8\text{-}173.3}{214.8\text{-}178.7}$	$\frac{179.01\text{-}173.3}{208.61\text{-}178.7}$	23%	18%
Dentin-Molar mesial root	$\frac{192.6\text{-}159.6}{196.4\text{-}142.5}$	$\frac{196.4\text{-}159.6}{209.0\text{-}142.5}$	61%	55%
Cementum-Molar distal root distal surface	$\frac{45.9\text{-}35.7}{83.4\text{-}19.8}$	$\frac{68.0\text{-}35.7}{120.0\text{-}19.8}$	16%	32%
Cementum-Molar mesial root distal surface	$\frac{42.3\text{-}37.4}{61.7\text{-}35.1}$	$\frac{51.0\text{-}37.4}{94.5\text{-}35.1}$	18%	22%
Cementum-Molar distal root mesial surface	$\frac{56.3\text{-}39.8}{86.4\text{-}25.2}$	$\frac{50.4\text{-}39.8}{137.9\text{-}25.2}$	27%	9%
Cementum-Molar mesial root mesial surface	$\frac{45.5\text{-}24.3}{81.0\text{-}11.7}$	$\frac{41.0\text{-}24.3}{107.5\text{-}11.7}$	30%	17%

x_1—Value of measurement after 1 week of feeding.
x_2—Value of measurement after 2 weeks of feeding.
x_3—Value of measurement after 3 weeks of feeding.

The periodontal membrane of the experimental animals was narrower than normal. It had increased during the experimental period at a significantly smaller rate of increase than in the control animals. Measurements were taken at 13 sites, and their average dimension showed an 11 per cent decrease in width from the normal. At 7 of these sites the difference was demonstrated to be statistically significant at the 1 per cent level of confidence. In the other 6 sites the difference was not statistically significant. In the control animals, the periodontal space facing the distal surface of the roots was wider than that facing the mesial surface. In the experimental animals, the periodontal space was wider on the mesial surface of the roots. In the experimental animals, the widest portion was located at the apex while the widest portion in the control animals was observed at the alveolar crest. The narrowest portion in both control and experimental animals was located at the bifurcation.

The most significant and consistent changes in the periodontal membrane were seen in the structure of this tissue, which underwent partial hyalinization (Figs. 7, 8, and 9). After the first week of the experimental diet the fibroblasts appeared swollen and separated by a homogeneous eosin-stained material,

resembling hyaline. The principal fibers were still intact, running from the surface of the alveolar process to a more apical area at the surface of the cementum. After 2 and 3 weeks of the experimental diet, the fibroblasts were arranged in rows separating hyaline lakes which had increased in size giving the entire periodontal membrane a striated appearance (Fig. 7).

After 2 weeks, the hyaline layers resembled osteoid tissue and the cells separating these layers resembled osteoblasts. The hyaline layers were principally located in the central portion of the periodontal membrane, being bordered mesially and distally by connective tissue. However, in some instances, they extended from the surface of the cementum to the surface of the alveolar bone. The hyaline layers and the rows of cells formed an angle of about 45 degrees, inclining from the surface of the roots to a more apical area at the surface of the alveolar bone. In sections impregnated with silver, fibrils could be observed entering the border areas of the homogeneous material and connecting one hyaline layer with the next, running between the cells. Only a few fibers could be traced entering into the hyaline lakes. These fibers ran in a direction similar to that seen in normal animals. The rows of osteoblast-like cells between the hyaline lakes appeared in palisade arrangement. The cells were of narrow cylindrical or spindle shape with their long axis parallel to the course of the principal fibers. The eccentric nuclei were intensely stained with hematoxylin and the cytoplasm showed a basophilic tinge. Some nuclei were located close to the coronal surface, others were close to the apical surface of the hyaline layers (Fig. 8). Occasionally some cells appeared trapped within the hyaline masses.

After 3 weeks of the experimental diet, there appeared, in several areas of the hyaline layers, granules which were stained with hematoxylin. The cytoplasm of the cells lining the hyaline layers showed granules which stained a purple hue. In some cells the cytoplasm contained small vacuoles (Fig. 8). In areas free of the hyalinized layers, signet ring cells were seen throughout the periodontal membrane. They contained clear cytoplasm and a compressed nucleus to one side.

There were also numerous large mononuclear phagocytic cells and some young as well as older typical fibroblasts. Epithelial remnants were present after the first week of feeding but could not be observed after the second and third weeks.

After 1 week on the experimental diet, the interdental alveolar septum was trapezoid to rectangular in shape in mesiodistal sections and consisted of spongy bone trabeculae. The marrow spaces of the gingival half of the septum were slightly enlarged compared to the controls. Some of the trabeculae were bordered by osteoclasts, which indicated active resorption. This was the initial stage in the development of osteoporosis of the alveolar process.

After the second and third weeks of the experimental diet, the bone marrow spaces of the gingival half of the septum were further increased in size (Figs. 6 and 7).

In most control animals there was active apposition on the alveolar walls facing the mesial surface of the roots, at the alveolar crest and at the alveolar

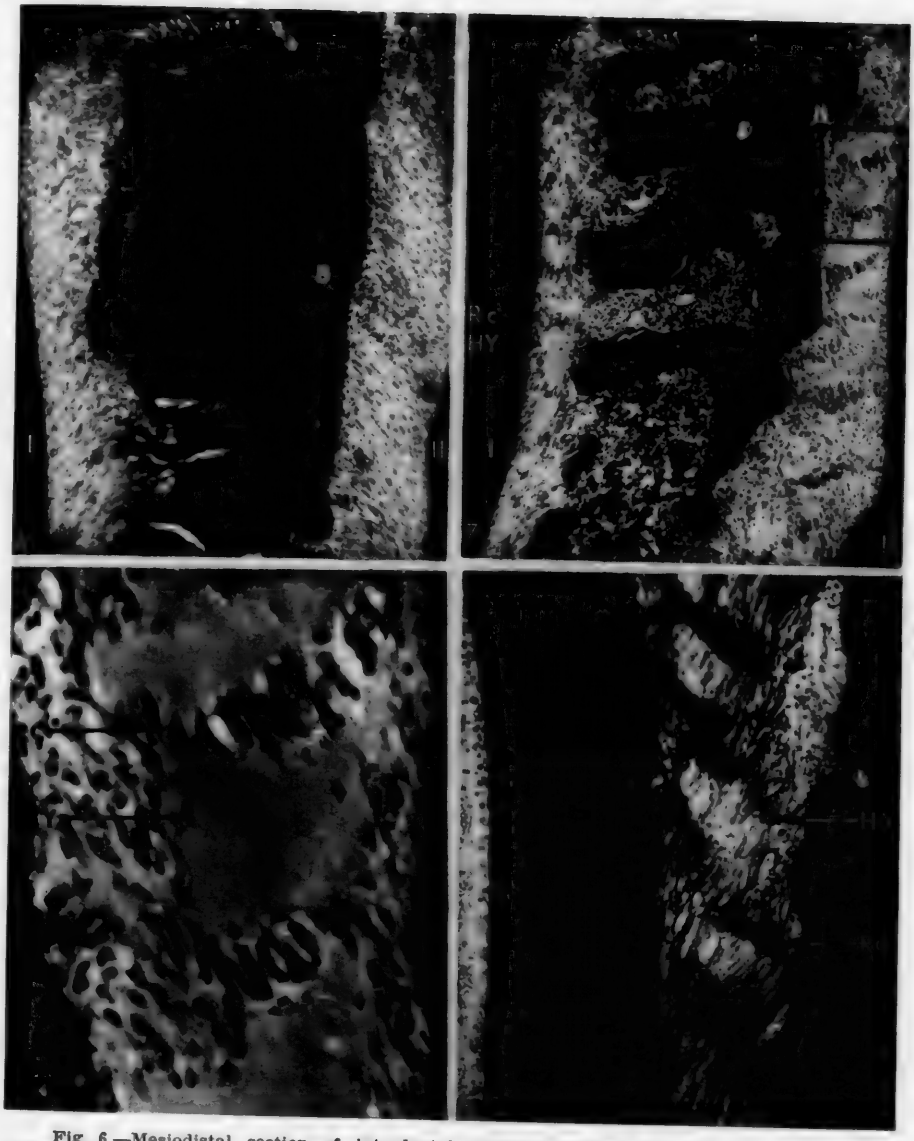


Fig. 6.—Mesiodistal section of interdental alveolar septum and periodontal membrane between the upper first (*I*) and second (*II*) molars of control animals, 43 days of age, fed a 50 per cent edible pea diet for 3 weeks. Principal fibers run from the surface of the alveolar process to a more apical area at the surface of the cementum, the marrow spaces are small, and bundle bone is wide at *B*. (Hematoxylin and eosin stain; orig. mag. ×115.)

Fig. 7.—Mesiodistal section of the interdental alveolar septum and periodontal membrane between the upper first (*I*) and second (*II*) molars of experimental animals, 43 days of age, fed a 50 per cent sweet pea diet for 3 weeks. Note hyaline layers separated by palisade arranged cells in the periodontal membrane, enlarged marrow spaces in the alveolar septum, and the lack of bundle bone formation. *Rc*—rows of palisade cells, *HY*—hyalinization. (Hematoxylin and eosin stain; orig. mag. ×115.)

Fig. 8.—Photomicrograph of area *A* in Fig. 7. Note hyaline layers separated by palisade arranged cells in the periodontal membrane. *RC*—rows of palisade cells, *HY*—hyalinization. (Hematoxylin and eosin stain; orig. mag. ×560.)

Fig. 9.—Photomicrograph of a mesiodistal section of the periodontal membrane of an experimental animal, 43 days of age, fed a 50 per cent sweet pea diet for 3 weeks. Note the large numbers of fibers in areas of cells (*Rc*) and the few fibers in areas of hyalinization (*HY*). (Mallory stain; orig. mag. ×270.)

fundus, and resorption was present on the alveolar walls facing the distal surfaces of the roots. In these areas both resorption and apposition were reduced or absent in the animals fed sweet peas for a period of 2 and 3 weeks, apparently a sign that the physiologic eruption and distal drift of the molars were arrested. An exception occurred at the alveolar crest, where apposition was still taking place, although in reduced amounts.

The distance between the alveolar crest and the cemento-enamel junction increased with age in the control animals. In the experimental animals, this distance had decreased by 39 per cent in the period of observation. At the mesial side of the second molar, the distance between crest and junction was demonstrated to be significantly different in the control and experimental animals at the 1 per cent level of confidence. At the distal side of the second molar this distance was decreased also, but the difference between control and experimental animals was not statistically significant. The reduction in the distance between the alveolar crest and the cemento-enamel junction apparently indicated an arrest of eruption concomitant with a slight progressive apposition of bone at the alveolar crest.

In the control animals, the distances between the alveolar crests and the cemento-enamel junctions were greater on the mesial than on the distal surfaces of the teeth. This was also found in the experimental animals after the first week of feeding. In animals fed for 2 and 3 weeks, the normal relationship was reversed. The distance was smaller on the mesial than on the distal surface, indicating a distal tipping in the control molars and a mesial tipping in the experimental molars.

The epithelium of the gingiva and the epithelial attachment, which was confined to the crowns of the teeth, did not show any changes during the experimental period. However, slight changes could be observed in the gingival connective tissue after 1 week of the experimental feeding. The number of fibroblasts was decreased, the collagen fiber bundles were swollen and appeared homogeneous, showing hyalinization. These changes did not increase with prolonged experimental feeding.

Upper incisors: The changes in the dentition and supporting structures of the incisor were similar in character but of much lesser degree than the changes of the molars.

The pigmentation of the enamel was normal. The enamel epithelium extended over the entire intra-alveolar labial portion of the incisor. The enamel organ in the experimental animals showed edema and vacuolization in the papillary layer. Edema in the adjacent connective tissue led to the formation of small round spaces between the cells of the papillary layer. The thickness of the enamel matrix showed a progressive increase with age in both control and experimental animals. However, the thickness of the enamel matrix at the level of completed formation, where the enamel matrix had been dissolved during decalcification, was greater by 10 per cent in the experimental animals (Table III). This was demonstrated to be statistically significant at the 1 per cent level of confidence.

TABLE III

DIFFERENCES IN HISTOLOGIC MEASUREMENTS ON THE INCISORS IN EXPERIMENTAL AND CONTROL ANIMALS AFTER 3 WEEKS ON THE EXPERIMENTAL DIET

SITE	DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL MEASURES, IN PER CENT OF THE CONTROL MEASUREMENT (%)
1. Enamel matrix	+10
2. Dentin	+10
3. Cementum	+11
4. Fundic connective tissue	+14

The pulp showed no observable changes from normal.

After one week of experimental feeding, the dentin in the anterior half of the incisor formed during the experimental period showed interglobular dentin indicating hypocalcification. The predentin layer next to the intermediate dentin contained a few isolated globules which stained similar to the dentin.

The thickness of the labial dentinal wall at the level of completed formation of the enamel matrix increased with the duration of the experimental period. In the experimental animals there was a 10 per cent increase in the width of the dentin (Table III). This was found to be statistically significant at the 1 per cent level of confidence.

The predentin was of the same thickness in control and experimental animals. The thickness of the lingual dentinal wall in control and experimental animals was consistently smaller than that of the labial dentinal wall, but was greater in the experimental animals than in the controls. The greater thickness of both the labial and lingual dentinal walls in the experimental animals was apparently due to a decreased rate of eruption.

The cementum consisted of a calcified layer with an adjacent thin layer of cementoid, which contained a few isolated cells. There was a gradual increase in the thickness of cementum of the experimental animals, which after 3 weeks of feeding was 11 per cent greater than in the controls (Table III). The increase occurred in all three areas measured, but was not statistically demonstrated. The tendency toward an increase in thickness of the cementum is apparently also due to a retardation in eruption.

The width of the periodontal membrane increased during the experimental period. However, in the experimental animals the increase in width was smaller at all sites measured. The experimental measurements averaged 9 per cent less than the controls, but the difference was not statistically significant. In experimental animals the narrowest portion was located near the fundus, whereas in the control animals it was located at the alveolar crest. The widest portion in the experimental and control animals was observed at the middle of the incisor.

After 1 week of the experimental diet no qualitative changes were observed. Mitoses in the basal portion of the periodontal membrane appeared to be more numerous in the experimental than in the control animals. The 3 typical layers of the ligament were seen in both groups of animals.

After 2 weeks of the experimental diet, in most specimens the periodontal membrane still showed 3 distinct zones but the cells of the intermediate plexus appeared disorientated. There were clustering and clumping of fibroblasts with hyalinization of collagen fiber bundles and the development of small hyaline lakes. A few areas showed continuous fibers running from the alveolar bone to cementum with loss of the 3 distinct zones.

After 3 weeks of the experimental diet the cells of the intermediate plexus were grouped into strands in the center of this layer. They had oval nuclei with 2 prominent dark-staining nucleoli, each. On either side of the strands of cells, clear zones were present which were composed of hyalinized fiber bundles. The hyalinization was more pronounced in the incisal third. In some cells, the nuclei were flattened, without nucleoli, and showed a wavy appearance. The blood vessels throughout the ligament were dilated.

The width of the labial alveolar periosteum increased during the experimental period in both control and experimental animals. The narrowest portion was located at the middle of the incisor. In the 3 areas measured no consistent difference existed between control and experimental animals. The labial alveolar periosteum showed focal areas of edema after the first, second, and third weeks of the experimental feeding, which were most prominent in the incisal third of the premaxillary portion. There was perivascular hyalinization in the zone close to the alveolar bone, and also hyalinization in locations distant from blood vessels. A decreased number of cells with vacuolization was present in the papillary zone adjacent to the enamel organ.

The width of the fundic connective tissue of the periodontal membrane increased during the experimental period. In the experimental animals the rate of growth was greater. It showed a 14 per cent increase in width which was statistically significant (Table III). The fundic portion of the periodontal membrane showed only slight hyperemia after 2 and 3 weeks on the experimental diet.

After the first week of the experimental diet, the alveolar bone showed no changes from the controls. After the second and third weeks of the experimental diet, the premaxillary portion of the labial alveolar bone exhibited resorption, which was not observed in the control animals.

Histologic changes in the mandibular joint and condyle: The condyle of the growing mandible of control animals consists of 3 structures: a layer of dense fibrous tissue, a layer of hyaline cartilage, and spongy bone. The peculiar mode of growth of the cartilage in the mandibular condyle permits the distinction of several layers which, in part, correspond to similar layers in the epiphyseal and articular cartilage of tubular bones and, in part, are specific for the mandible.

Subjacent to the dense fibrous layer which is in direct contact to the lower surface of the interarticular disk is found a layer of loose connective tissue, from which the appositional growth of the condylar cartilage occurs. This layer blends gradually into a transitional layer, in which young mesenchymal cells

differentiate into chondroblasts. In the cap of hyaline cartilage, the chondroblasts gradually grow until they reach their definitive functional size. In the next deepest layer, the cells begin to degenerate and gradually swell by intracytoplasmic and intranuclear edema to almost 3 times the size of fully grown chondrocytes. At the same time the bars of intercellular substance separating these cells become thin. Then follows, in consequence of progressive cell degeneration, the calcification of the intercellular substance and finally its destruction by cells of the proliferating connective tissue that may be called osteoclasts or chondroclasts (Figs. 10 and 12).

Thus, the layers forming the condyle of the growing mandible can be enumerated as follows: (1) the layer of dense articular fibrous tissue, (2) the layer of transition between the fibrous tissue and cartilage or the layer of appositional growth of the cartilage, (3) the layer of chondrocyte growth, (4) the layer of hydropic degeneration of chondrocytes, (5) the layer of calcification of intercellular substance, and (6) the layer of destruction of the cartilage. Subjacent to all these layers is found the primary spongiosa of the bony condyle (Fig. 10).

The first changes in the mandibular articulation occurred already after one week of feeding. The condyle was reduced in mediolateral direction due to increased resorption of cartilage and primary spongiosa at its medial border (Fig. 11). While the fibrous covering of the condyle remained unaltered, the layer of transition between the dense fibrous tissue and cartilage had become edematous and appeared sharply demarcated against the hyaline cartilage. The hyaline cartilage itself was reduced in thickness and showed signs of excessive interstitial growth. Territorial groups of cells were seen throughout the cartilage. In each group the cells lay close together, but each group was separated from the next by a fairly thick layer of intercellular substance. Hydropic degeneration and swelling of the cells did not take place, although signs of calcification, characterized by increased basophilia, were visible in the deepest layers of the cartilage. The primary spongiosa was well calcified; very little if any osteoid could be observed. The trabeculae were irregular and showed signs of osteoclastic resorption. The marrow spaces in the primary spongiosa were much wider than in the controls. At the edge of the roof of the mandibular articulation (squamosal bone) osteophytic bone apposition had slightly deepened the articular groove.

After 2 weeks on the experimental diet, the changes in the condyle had progressed to a marked degree. The remnants of the cartilage were restricted to the superolateral aspect of the mandibular head. The cartilage was well demarcated against the surrounding connective tissue and showed signs of interstitial growth. Only at the medial edge of this cartilage did the chondrocytes show signs of edema. The flattened medial surface of the condyle was covered by a thick layer of proliferating connective tissue that extended ventrally along the neck and part of the ramus between the bone and bundles of the external pterygoid muscle. The primary spongiosa was quite irregular, the marrow spaces very wide (Fig. 13).

Fig. 10.—Photomicrograph of mediolateral section of the condyle of a control animal, 29 days of age, fed an edible pea diet for 1 week. *1*—Layer of articular fibrous tissue. *2*—Layer of transition. *3*—Layer of chondrocyte growth. *4*—Layer of hydropic degeneration. *5*—Layer of calcification. *6*—Layer of destruction. *7*—Primary spongiosa. *EP*—external pterygoid muscle. *D*—interarticular disk. *ZS*—zygomatic process of squamosal bone. (Hematoxylin and eosin stain; orig. mag. ×60.)

Fig. 11.—Photomicrograph of mediolateral section of the growth cartilage of mandibular head of experimental animal, 29 days of age, fed a 50 per cent sweet pea diet for 1 week. Note the change in shape of the condyle as compared with Fig. 10, and the reduction of the hyaline cartilage (*CC*) with signs of interstitial growth. *CC*—condylar cartilage, *S*—squamosal bone, *EP*—external pterygoid muscle, *D*—interarticular disk. (Hematoxylin and eosin stain; orig. mag. ×60.)

Fig. 12.—Photomicrograph of mediolateral section of the growth cartilage of mandibular head of 36-day-old control rat fed a 50 per cent edible pea diet for 2 weeks. (Hematoxylin and eosin stain; orig. mag. ×60.)

Fig. 13.—Photomicrograph of mediolateral section of the growth cartilage of the mandibular head of an experimental animal, 36 days of age, after 2 weeks on a 50 per cent sweet pea diet. Note interstitial growth in the hyaline cartilage (*CC*), and edema of chondrocytes (*I*) only at the medial edge of the hyaline cartilage. *D*—interarticular disk, *ZS*—zygomatic process of squamosal bone, *BM*—hematopoietic bone marrow, *S*—squamosal bone. (Hematoxylin and eosin stain; orig. mag. ×60.)

Figs. 14*A* and 14*B*.—Photomicrograph of mediolateral section of mandibular ramus of control (*A*) and experimental (*B*) animals, 43 days of age, after 3 weeks of their respective diets. Note exostoses at the edge of the articular roof (*1*), along the medial border of the ramus (*2*), and at the angular process (*3*). *T*—temporal muscle, *AP*—angular process, *EP*—external pterygoid muscle, *CC*—condylar cartilage, *m*—masseter muscle, *S*—squamosal bone, *IP*—internal pterygoid muscle, *ZS*—zygomatic process of squamosal bone. (Hematoxylin and eosin stain; orig. mag. ×10.)

Fig. 15.—Photomicrograph of mediolateral section of mandibular ramus of experimental animal, 43 days of age, after 3 weeks of the sweet pea diet. Note the total disappearance of the growth cartilage (*CC*), replacement of the temporal muscle by connective tissue (*1*), new bone formation at the edge of the articular roof (*2*), along the ramus (*3*), and angular process (*4*). Higher power of areas *A*, *B*, *C* are shown in Figs. 18, 19, 20, 21, respectively. (Hematoxylin and eosin stain; orig. mag. ×10.)

Fig. 16.—Photomicrograph of mediolateral section of the growth cartilage of the mandibular head of a 43-day-old experimental animal after 3 weeks on the sweet pea diet. Note irregular shape of the condyle, reduction in the thickness and interstitial growth in the cartilage. *CC*—condylar cartilage, *S*—squamosal bone, *BM*—hematopoietic bone marrow. (Hematoxylin and eosin; orig. mag. ×60.)

Fig. 17.—Photomicrograph of mediolateral section of the mandibular head of a 43-day-old experimental animal after 3 weeks of the sweet pea diet. Note total disappearance of growth cartilage and replacement by proliferating connective tissue and few trabeculae of mature type bone at *CC*. *S*—squamosal bone, *ZS*—zygomatic process of squamosal bone, *D*—interarticular disk, *ep*—external pterygoid muscle. (Hematoxylin and eosin stain; orig. mag. ×60.)

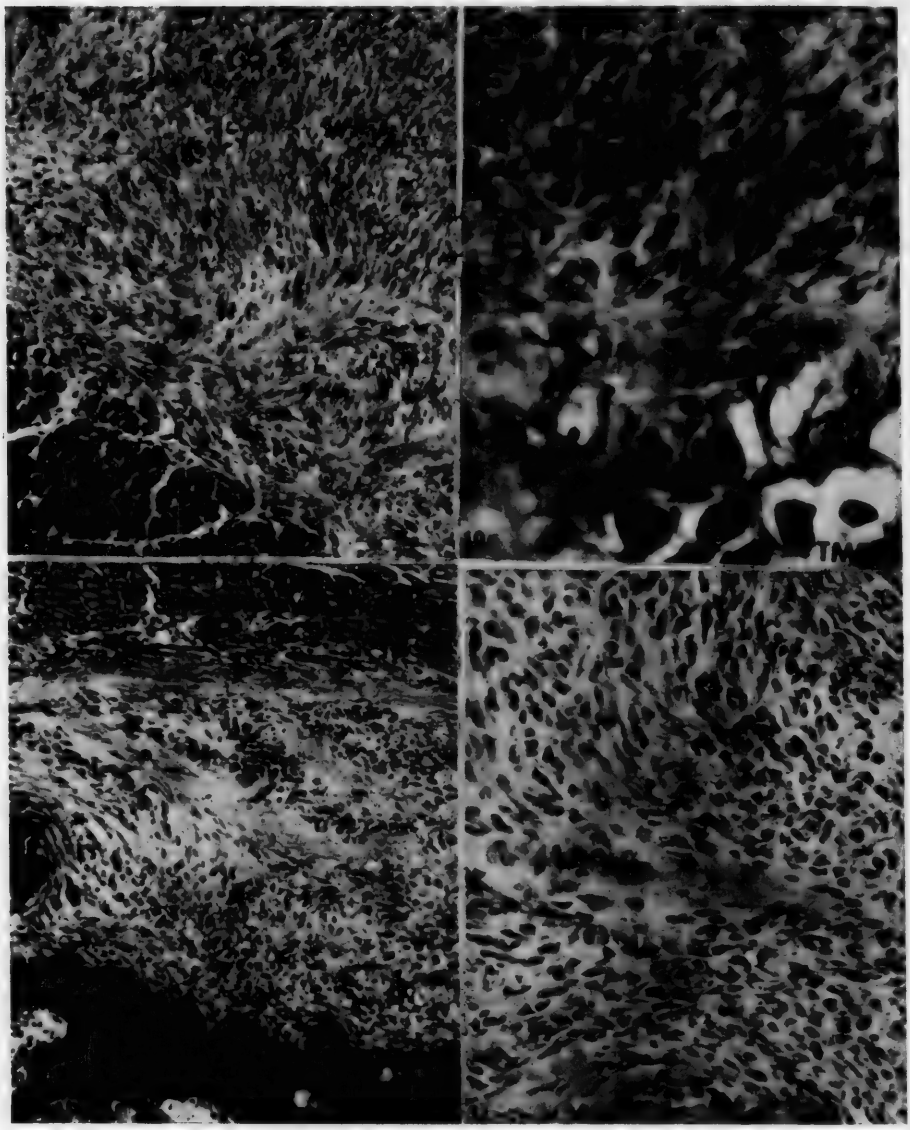


Fig. 18.—Photomicrograph of area *A* in Fig. 15. Note proliferating connective tissue replacing and invading the temporal muscle. *TMP*—temporal muscle. (Hematoxylin and eosin; orig. mag. ×270.)

Fig. 19.—Photomicrograph of area *A* in Fig. 18. Note proliferating connective tissue replacing and invading the temporal muscle. *TMP*—temporal muscle. (Hematoxylin and eosin; orig. mag. ×560.)

Fig. 20.—Photomicrograph of area *B* in Fig. 15. Note hyalinization of the connective tissue. *MPR*—medial plate of ramus, *EP*—external pterygoid muscle. (Hematoxylin and eosin stain; orig. mag. ×270.)

Fig. 21.—Photomicrograph of area *C* in Fig. 15. Note beginning calcification in the connective tissue replacing the roof of the articular groove. (Hematoxylin and eosin; orig. mag. ×400.)

After the third week on the experimental diet, the changes were not nearly as uniform. Two types of reaction could be differentiated. One type, occurring in 2 of 6 animals, can be classified as progress in the changes previously described. A rather large, irregularly outlined plate of hyaline cartilage was present, again showing signs of continuing interstitial growth and calcification, but not any clear signs of degeneration of the chondrocytes (Fig. 16). The remaining animals showed a complete disappearance of the growth cartilage. The condyle was distorted and consisted only of loose proliferating connective tissue and few bone trabeculae (Fig. 17).

The formation of exostoses was studied at the angular process, where the internal pterygoid muscle is attached to the medial surface, at the medial surface of the ramus below the mandibular neck in the area of attachment of the external pterygoid muscle, and at the edge of the articular roof of the mandibular articulation in the area of attachment of the temporal muscle.

After 1 week of experimental feeding, connective tissue had proliferated at the sites of tendons and had partly invaded the muscle tissue. The connective tissue cells were of cylindrical or tapered shape, the nuclei were located eccentrically at one pole of the cell and were hyperchromatic. In some areas, connective tissue cells appeared to be arranged in a palisade manner (Figs. 20 and 21).

After 2 and 3 weeks of the experimental diet, hyalinization occurred between the cells in palisade-like arrangement (Fig. 20). In some foci of hyalinization, areas of increased basophilia could be observed, apparently a sign of calcification (Fig. 21). The cells adjacent to the hyalinized areas seemed to have differentiated into osteoblasts. After 3 weeks, bone trabeculae of immature type of bone had developed in this proliferating connective tissue. At the angular process the trabeculae were arranged irregularly, whereas at the edge of the articular roof the bone trabeculae showed an osteophytic arrangement (Figs. 14, *B* and 15). In this area, bone apposition had progressed so far that the roof of the mandibular articulation continued into a long plate which was thickened at its edge and which covered the upper one third of the ramus (Figs. 14, *B* and 15).

DISCUSSION

The changes in the masticatory apparatus of rats fed sweet peas can be classified under 4 headings: (1) retardation in growth, (2) changes at sites of insertion of ligaments and tendons, (3) resorption of bone and cartilage, and (4) etiology and pathogenesis.

Retardation in Growth.—The retardation in growth of the experimental animals was apparent from their smaller weight, smaller over-all length of the cranium and mandible, and lower height of the mandibular body. The extra-alveolar portions of the upper and lower incisors were progressively and markedly reduced. Since it can be assumed that attrition progressed at physiologic rate, the reduction in the extra-alveolar portion of the incisors might have been caused by a retardation of eruption. Sicher[26] has shown that the eruption

of the incisors is mainly due to growth of the pulpal connective tissue. Therefore, the retardation of eruption appears to be caused by an impairment of connective tissue growth. Not only is the eruption of the incisors retarded, but also the eruption of the molars, as suggested by the distortion of the roots, and by the reduction in the distance between the cemento-enamel junction and the crest of the alveolar process.

In the control animals, the diastema between the upper incisors and upper molars and between the lower incisors and lower molars increases parallel with the increase in the length of the jaws. The increase in length of the diastema is partly accomplished by a distal drift of the molars, partly by growth of the alveolus of the lower incisor, and in the maxilla by growth at the maxillary-premaxillary suture. The physiologic distal drift is characterized by apposition on the mesial surfaces and by resorption of the alveolar socket on the distal surfaces of the root. In the experimental animals, the diastema increased less than in the control, indicating a retardation in the above-mentioned growth processes.

The retardation of the apposition of the mesenchymal calcified tissues of the teeth was indicated by a reduction in the width of the dentinal wall of the upper molars and in the width of the cellular cementum at the apical third of the molar roots. The width of the acellular cementum in the coronal two thirds of the molar roots was not reduced because in this area practically no growth was occurring in normal animals. The cementum and dentin of the incisors was increased in width, which is not due to stimulated growth in the experimental animals but to the prolonged apposition of cementum and dentin in consequence of the retardation of the eruption of these teeth.

Formation of Exostoses.—The formation of exostoses occurred at the cranium and mandible only in areas of muscle attachment. As early as 1 week of the experimental diet the tendinous insertions and adjacent muscles were replaced by proliferating young connective tissue. Shortly after replacement, and to some extent simultaneously, some of the young connective tissue cells differentiated into osteoblasts and formed irregularly arranged trabeculae of immature type of bone. At a later stage, the connective tissue between the bone trabeculae assumed the character of fibrous bone marrow. Similar changes have been described at the insertion of muscles in other parts of the skeleton as a result of feeding sweet peas.[7a, 12, 16, 35]

Changes in the Periodontal Membrane.—The changes in the periodontal membrane were unlike any changes in the periodontal membrane observed under other experimental conditions or in human pathology. They are initiated by changes comparable to the early changes at the insertions of tendons. The principal fibers of the periodontal membrane may be regarded as ligaments and are arranged in the lines of tension. After replacement of the principal fibers of the periodontal membrane by loose connective tissue, the forces applied to the tooth are no longer transformed into tensile forces but exert pressure on the periodontal tissues. In response to this change in functional load, osteoblasts

differentiate in the loose connective tissue and form trabeculae of osteoid tissue that are arranged in the direction of pressure and, thus, at right angles to the direction of the fibers of the normal periodontal membrane.

Resorption of Bone and Cartilage.—Resorption of bone leading to an increase in the bone marrow spaces (osteoporosis) has been observed in the alveolar process and in the ramus of the mandible. At the anterior border between the neck of the mandible and the condyle the proliferating connective tissue invaded the growth cartilage, leading to its destruction and finally replacement by young connective tissue. In some areas of the replacing connective tissue, immature bone formation could be observed.

Etiology and Pathogenesis.—It has been shown that the active toxic factor in the sweet pea is beta-aminopropionitrile.[22] It has been tentatively concluded that the primary change occurs in the cementing substance of the mesenchymal tissues, possibly by affecting production of mucopolysaccharides.[8, 16] The changes in the periodontal membrane of the second molar were severe, whereas the changes in the alveolar periosteum and periodontal membrane of the incisors were extremely minute. The rat incisor is a rather rapidly and continuously growing and erupting tooth and, therefore, it can be assumed that the turnover in the supporting tissues of this tooth is greater than in the periodontal membrane of the molars. This difference would suggest therefore that the older connective tissue is more susceptible than young connective tissue to the toxic factor in sweet peas and that the cause for the changes observed is primarily a destruction of the mucopolysaccharides rather than a decreased production.

The proliferation of the connective tissue, which in the area of the insertion of tendons assumed almost neoplastic qualities, invading muscle tissue and leading to the degeneration of muscle cells, could be regarded as a reaction to the degenerative changes of the cementing substance.

The changes in the hyaline cartilage of the condyle, which were similar to the changes observed in the cartilage of other joints[12, 16] can be explained also by degeneration of the cementing substance. The rents observed by Ponseti[16] in the cartilage of the knee joint and other joints have not been observed in the growth cartilage of the condyle.

Whether the retardation of growth of the skull, mandible, incisors, and molars is also caused by regeneration of the cementing substance of the mesenchymal tissues in the growth centers (sutures, cartilage, pulp) or by a more general toxic effect could not be decided by the present study.

SUMMARY AND CONCLUSIONS

This study is based on the radiographic and histologic investigation of the molar and incisor, their supporting structures, and the mandibular condyles of 14 experimental and 14 control albino rats, ranging from 22 to 43 days of age. The experimental animals were fed a balanced diet, to which 50 per cent ground sweet peas were added. The control animals received the same basic diet to which 50 per cent edible pea meal was added.

The most striking changes were destruction of the condyle, development of exostoses at the insertion of tendons, and replacement of the principal fibers of the periodontal membrane by alternating layers of osteoid tissue and rows of cells resembling osteoblasts. These layers were orientated along the trajectories of pressure.

In addition, there were changes in growth manifested by decrease in body weight, retardation in growth of cranium and mandible, incisor and molar, dentin and cellular cementum of molars. Secondary to changes in growth, physiologic drift of molars was retarded and the cutting surfaces of incisors notched.

It is concluded that the pathologic changes observed in the present study can be explained by destruction of the cementing substance of mesenchymal tissues by the toxic factor of sweet peas.

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AN ANALYSIS OF STRESSES IN A DENTAL AMALGAM RESTORATION

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ALTHOUGH there has been a general agreement concerning the over-all design of proximo-occlusal amalgam restorations for the primary molar teeth, differences of opinion do exist concerning certain aspects of this design; particularly in the category of resistance form. It was the general purpose of this investigation to evaluate this restorative design in anticipation of resolving these differences. Since the primary molar teeth differ considerably in morphology, it was thought desirable to evaluate one specific design in order to define more precisely the problem to be considered. A disto-occlusal restoration in a primary mandibular first molar with incipient decay was chosen. In Fig. 1, the cavity preparation for this restoration from a distolingual viewpoint is shown. The main retentive features of this restoration consist of an undercut occlusal dovetail, a proximal box wider at the gingival than at the occlusal, and a proximal retention groove.

The fundamental concept of resistance form is based on the reaction within the restoration and remaining tooth structure to occlusal loading; the nature of this reaction being the development of internal stress. When the internal stress developed exceeds certain limits, structural failure will result. It is significant to recognize that both the restoration and remaining tooth structure must resist structural failure if the restorative attempt is to be successful. The design of the cavity preparation limits the structural effectiveness of both the restoration to be placed and the remaining tooth structure. Providing bulk for the restoration can only be done at the expense of tooth structure and, conversely, conservation of tooth structure can be accomplished only at the expense of the restoration. In evaluating internal stress, then, the basic problem would be to determine the best possible configuration of the prepared cavity in keeping with the distribution and magnitude of the stresses induced in the tooth structure as well as the prosthesis.

There are several factors which influence the stress response, the most significant being the character of the occlusal loading and the shape or design of the restoration.

FACTORS INFLUENCING STRESS RESPONSE

Occlusal Loading.—An analysis of the character of the occlusal loading may start at an extreme limit where the restored tooth is out of occlusion and, since no forces are applied, no stresses result. This, of course, would prevent

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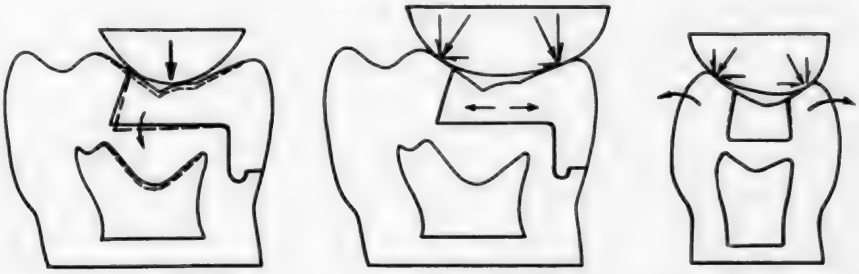
the normal physiologic function of the tooth in the dental arch. If the restoration only were out of occlusion, the surrounding tooth structure would have to support a proportionally greater amount of load and its resistance to failure would decrease; and the reverse would exist if only the tooth were out of occlusion. It would appear desirable from a functional standpoint to bring the entire occlusal area of both the tooth and restoration into occlusion. The actual morphology of the occlusal area of both the tooth in question and the

Fig. 1.—The disto-occlusal cavity preparation in a primary mandibular first molar from a distolingual viewpoint.



A. *B.* *C.*

Fig. 2.—Character of occlusal loading. *A*, Narrow opposing cusp (mesiodistal section). *B*, Wide opposing cusp (mesiodistal section). *C*, Wide opposing cusp (buccolingual section).

opposing cusp, as well as their relative intercuspation, will also determine the nature of the internal stress response. In Fig. 2, *A* and *B*, are shown 2 different loading conditions produced by 2 differently shaped opposing cusps viewed in a mesiodistal section through the tooth. In Fig. 2, *A* the loading is shown directed over the central pit and a bending of the restoration and tooth structure about the axiopulpal line angle may be anticipated. In Fig. 2, *B* is shown an occlusal contact arrangement which tends to separate the proximal

portion of the restoration from the occlusal dovetail. It is felt that this latter type of loading is responsible for the isthmus failures of the restoration as observed in clinical practice. Although two differently shaped opposing cusps are shown in these 2 cases, the small cusp could produce a similar loading situation as that shown for the wide cusp by contacting the marginal ridge either in a centric or protrusive position. In the buccolingual plane, the most deleterious loading situation would be that shown in Fig. 2, *C*, where the opposing cusp tends to bend the buccal and lingual walls of the tooth away from the restoration. It is in this particular section that the resistance of tooth structure to failure could be analyzed most propitiously.

Restorative Design.—There are 2 design features which provide resistance form for the restoration. One is the retentive grooves placed in the proximal box at the axiobuccal and axiolingual line angles and often extended across the gingival wall at the axiogingival line angle. These grooves help to prevent the proximal box from being displaced from the cavity preparation, and resistance is supplied by shear stresses acting across the area of the grooves where they join the main body of the restoration. The only differences of opinion which exist relative to this feature is the instrumentation necessary to make the groove. Brown[1] specifies a No. 33½ inverted cone bur, Ireland,[2] a No. ½ round bur, and Sweet,[3] the end of a No. 700 tapered fissure bur. The effect of these minor variations in groove design appear of little consequence in explaining the failures observed in practice.

It has been reasoned that since structural failure of the restoration most usually occurs at the isthmus, an increase in the area of this region should increase the resistance of the restoration to failure. Brown[1] specified a flat pulpal wall whose depth is defined by a plane passing through a point just below the dentinoenamel junction at the central pit and a width slightly greater than the large diameter of a No. 39 inverted cone bar (about 2.0 mm.). Ireland[2] specified a sloping pulpal wall which is rounded at the isthmus, thereby increasing the depth of the restoration over the flat pulpal wall depth of Brown's design. At the center of the curved wall, the increase in depth is equal to approximately 0.5 mm. The idea of the curved wall is to increase the depth of the pulpal wall yet at the same time avoid the distobuccal and distolingual pulp horns which lie close to the occlusal surface in this tooth. Ireland's isthmus width is specified as ¼ the buccolingual diameter of the crown which for the primary first mandibular molar would be approximately 1.8 mm. and is slightly less than Brown's width. Sweet[3] recommended a pulpal floor which parallels the contour of the occlusal morphology of the tooth and lies just beneath the dentinoenamel junction. His width at the isthmus is indicated as being ". . . the maximum width possible," and from the illustrations in his report, this width measures close to 2.5 mm. with due consideration for the average dimensions of the mandibular first molar.

Since failure of tooth structure would most likely occur in the buccal and lingual walls which have lost structural continuity as a result of cavity

preparation, any increase in width of the restoration will decrease the resistance to failure of these walls. The possibility of fracture exists as well as a bending of these walls relative to the restoration which would permit a separation of tooth and restoration. In addition, increasing the depth of the cavity preparation decreases the width of the dentin bridge overlaying the pulp chamber which may prove detrimental in some instances. Brown's preparation is most conservative of tooth structure, whereas, Ireland's design decreases the width of the bridge of dentin over the pulp, and Sweet's design decreases the width of the buccal and lingual walls of the tooth.

Although a direct comparison of these variations might provide the answer to the immediate problem of which of these designs is best, it was felt that an investigation of the fundamental mechanisms involved would not only allow for a rationalization of these design variations but, in addition, might yield design recommendations not heretofore considered.

Significant Stresses.—The logical approach to the analysis would be to determine the maximum tensile, compressive, and shear stresses in the entire structure, find where these occur, and relate them to the strength properties of the evolved structures. However, this burden may be considerably eased by utilizing auxiliary information which is available. In fact, this has been done already to some extent by choosing the isthmus area as the area for investigation on the basis of the observed clinical failures of the restoration and the buccal and lingual walls as the logical point of failure for tooth structure. In addition to this consideration the nature of the strength of the materials involved has considerable bearing on what stresses should deserve major consideration. Amalgam acts like a brittle material under the rapid rates of loading present during the application of occlusal forces. While the shear strength of amalgam is about one half its compressive strength the tensile strength is about one tenth its compressive strength.[4] It would be logical to give tensile stresses major consideration for this restorative material.[5]

The enamel offers little structural benefit once its continuity has been disturbed, largely due to the low resistance to fracture along planes parallel to the direction of the enamel rods. Dentin may be classified as a ductile material and, consequently, the evaluation of shear stresses would be most significant when considering vital tooth structure.[5]

METHOD OF TEST

Since optimum resistance form of both the restoration and remaining tooth structure is that which provides the most favorable internal stress response to occlusal forces, the technic of evaluation is necessarily one of analyzing the nature and magnitude of these internal stresses. On the basis of the nonuniformity of dental restorative design as well as the proximity of the applied forces to the points of stress evaluation, the method of photoelasticity was chosen as the method of stress analysis.

The general sequence of the photoelastic procedure consists of duplicating the shape of the actual structures in a photoelastic sensitive model material, loading this model in a manner similar to that which occurs in the original structures and then analyzing the stresses which develop in the model. These stresses may then be referred to the original structure. Since the photoelastic data yields information concerning only the maximum shear stresses in the structure, it is important that additional information be utilized in order to determine the maximum tensile stresses, especially since a brittle material is involved. This may be accomplished by the use of a lateral extensometer which measures lateral deformation of the model under load. The exact procedure used for a complete evaluation of stress and the fundamental principles of the technic are described in a previous report.[6]

Fig. 3.—Photoelastic testing model and mold for pouring supporting structure of gypsum.

The models used to investigate the stresses were chosen to be 8 times larger than the actual tooth for ease in testing. A mold of Plexiglas was made which consisted of two side pieces which sandwiched the photoelastic sensitive model (Catalin cast resin 61-893) in between (Fig. 3). An improved dental stone was poured into the mold to serve as the supporting structure. The intimate contact of support and model produced by this technic resulted in negligible stress concentrations at the interface. The restoration was extended over the entire tooth for facility in testing (Fig. 3). The stress response at the isthmus was found not to be influenced by this expediency.

The mold for the buccolingual section, used to evaluate the resistance of tooth structure, was constructed in a similar fashion. In this instance, however, the model was the tooth structure and the improved stone was poured into the space occupied by the restoration.

RESULTS AND DISCUSSION

As indicated previously, the procedure of analysis is to determine where the maximum tensile stress occurs in the amalgam restoration and the effect

that design variations have on this stress, thereby indicating the most favorable design. In a similar fashion the remaining tooth structure is analyzed where the maximum shear stress is of significance.

In Fig. 4 is shown the photoelastic stress pattern on a mesiodistal section through the tooth and restoration for an occlusal load of 75 pounds. Light lines were scribed every 2 mm. to fix the position of each point on the model. From this figure, the value of $\frac{p - q^*}{2}$ at any point in question was determined by determining the order of the interference bands passing through each point. $\frac{p + q}{2}$ was determined by measuring the lateral deformation (δ) at these same points. Simple algebra was then used to determine p and q individually. In Table I is shown a tabulation of these stresses along a vertical plane 4 mm. in from the axial wall. (This particular plane was taken to represent the isthmus position in the actual tooth. Since the models are an 8× magnification, this would be 4/8 = 1/2 mm. in the actual tooth.)

Fig. 4.—Photoelastic stress pattern for the restoration. Occlusal load of 75 pounds applied.

In Fig. 5, the restoration has been schematically drawn and the principal tensile stresses are indicated in vectorial representation. As can be seen, the tensile stresses act in a predominantly horizontal direction. Even more significant is the distribution and magnitude of these tensile stresses which are low at the pulpal wall but increase markedly and reach a maximum at the occlusal surface. On the basis of the behavior of amalgam under high rates of loading, the maximum tensile stress should set the criterion for failure and since this occurs at the occlusal surface, it is in this region that failure would be initiated. The stress in the region of the axiopulpal line angle would, therefore, not be indicative of restorative failure under the conditions of test as prescribed. This result provides an explanation for the unexpected result obtained in experiments by Ireland[2] whereby the rounding of the pulpal axial

*p = maximum principal stress (usually the highest tenaile stress).
q = minimum principal stress (usually the highest compressive stress).
$\frac{p - q}{2}$ = maximum shear stress.

TABLE I

DETERMINATION OF p AND q AT THE ISTHMUS

POSITION	n	$\frac{p-q}{2}$	δ	$\frac{p+q}{2}$	q	p	θ
0	0.68	119	–	–	–	–	0
2	1.00	175	–.12	– 77	–252	+ 98	– 5
4	1.04	182	–.04	– 26	–208	+156	– 5
6	1.12	196	–.03	+ 19	–177	+215	– 5
8	0.94	165	–.12	+ 77	– 88	+242	– 5
10	0.88	154	–.18	+116	– 38	+270	+ 7
11.4	1.17	205	–	+205	0	+410	+26

Position measured in millimeters from pulpal floor

n = fringe order

$\frac{p-q}{2} = 43.6\ n/t = \frac{43.6}{.249}\ n = 175\ n$

δ = lateral deformation

$\frac{p+q}{2} = -160\ \frac{\delta}{t} = \frac{-160}{.249}\ \delta = -644\ \delta$

θ = isoclinic (direction of principal plane)

t = model thickness

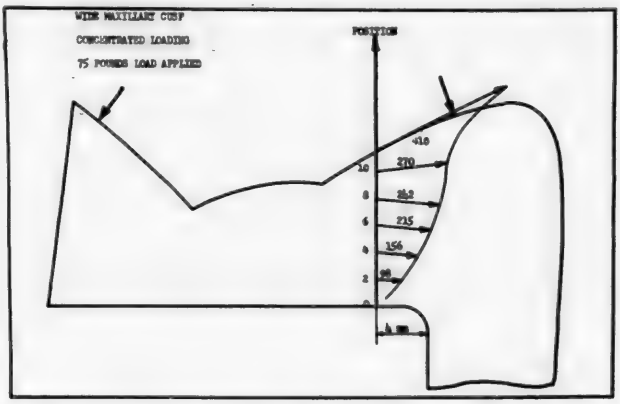


Fig. 5.—Schematic sketch indicating stress distribution and magnitude across the isthmus of the restoration.

line angle did not significantly increase the resistance of the restoration to failure. A rounding of this angle would tend to increase the depth of the restoration at this point which may be reflected in a slightly lowered tensile stress at the occlusal surface, but since the maximum tensile stress does not occur here, a pointed apex, although producing a stress concentration, would, therefore, not be significantly deleterious.

Since the most significant stress occurs at the occlusal surface, a series of experiments were run to determine where on the occlusal surface (in a mesio-distal direction) the maximum stress occurred and also which design feature would produce the most favorable stress response. The results of these tests are given in Table II. The designs are illustrated in Fig. 6 which also shows their respective photoelastic stress patterns under similar conditions of test.

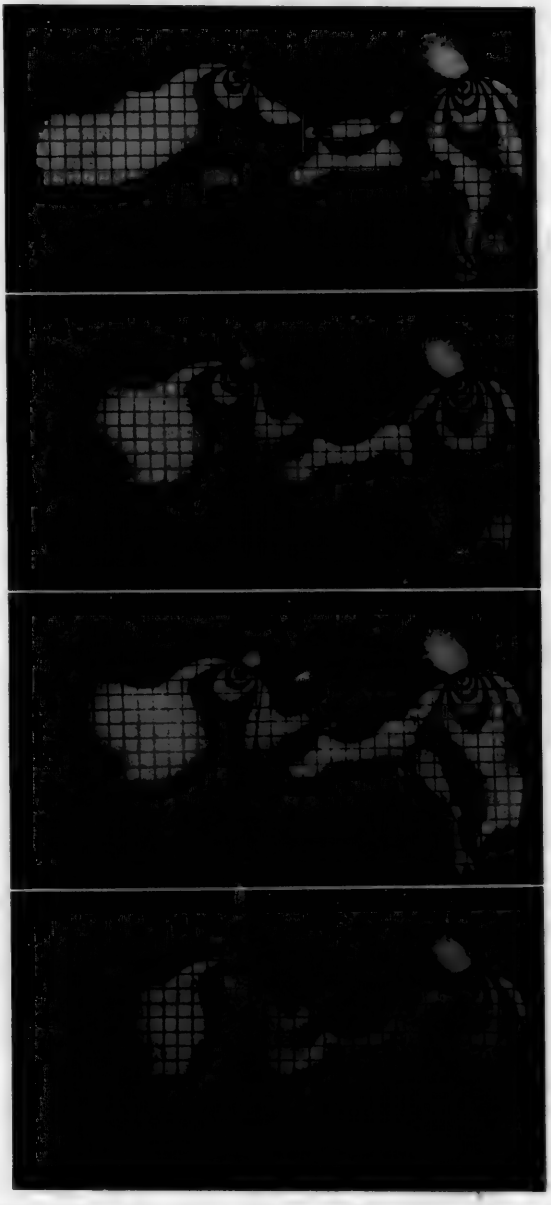


Fig. 6.—Restorative designs. Mesiodistal section. *A*, Flat pulpal wall. *B*, Sloping pulpal wall. *C*, Beveled pulpal wall. *D*, Sloping axial wall.

TABLE II

TENSILE STRESSES AT THE OCCLUSAL SURFACE FOR VARIOUS RESTORATIVE DESIGNS

RESTORATIVE DESIGN	POSITION FROM AXIAL WALL ON MODEL (MILLIMETERS)					
	2	4	6	8	10	12
Flat pulpal wall (Fig. 6, *A*)	320	410	470	960	590	560
Sloping pulpal wall (Fig. 6, *B*)	220	370	420	700	490	450
Beveled pulpal wall (Fig. 6, *C*)	190	320	370	690	460	400
Sloping axial wall (Fig. 6, *D*)	180	280	370	680	440	390

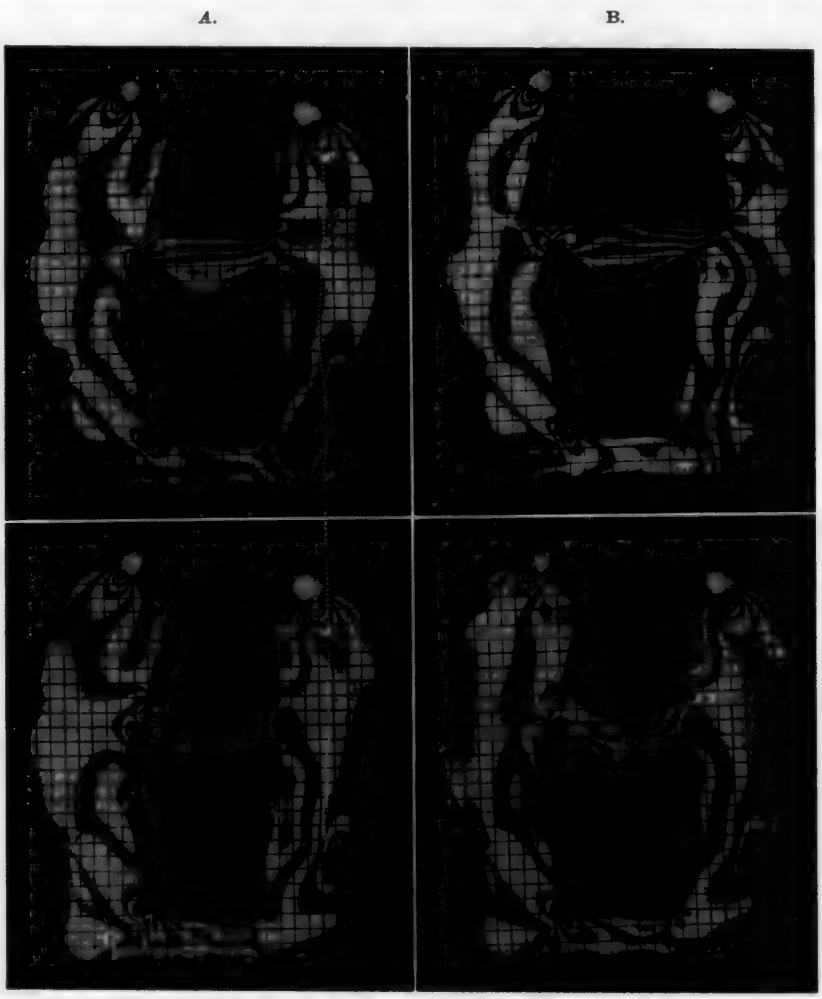


Fig. 7.—Restorative design. Buccolingual section. *A*, Flat pulpal wall, moderate width. *B*, Flat pulpal wall, increased width. *C*, Rounded pulpal wall, moderate width. *D*, Tapered pulpal wall, moderate width.

From these data it can be seen that the tensile stress increases as the distance from the axial wall increases. It would therefore be indicated to place the isthmus as close to the axial wall as possible.

At 8 mm., the stress is very high and then drops back at 10 mm. This high stress corresponds to a cuspal developmental groove which was carved in the restoration and duplicated in the model to correspond to the one in the original tooth structure. It appears contraindicated to include these grooves in the occlusal morphology of the restoration in this region of maximum tensile stress. Even a rough amalgam in this critical region may produce a stress concentration of deleterious magnitude.

The difference between the various designs indicates that a sloping pulpal wall is of greater advantage than the flat pulpal wall. This advantage is counteracted by the adverse stress developed in the tooth. A beveled pulpal wall is slightly better and the best situation occurs with a sloping axial wall and a flat pulpal wall. This last design is of still greater advantage since it is the most conservative of tooth structure.

The stress response in the tooth is shown in Fig. 7. In this section, since the shear stress within the tooth is of significance, the density of the dark bands is the criterion for evaluation. In Fig. 7, *A*, is shown a buccolingual section of the tooth and restoration with its corresponding stress pattern for a flat pulpal wall. In Fig. 7, *B*, is shown the effect of an increase in width of restoration and the stress is seen to increase in intensity. In Fig. 7, *C* and *D*, the effects of reducing the pulpal wall by rounding or tapering shows a very large increase in stress in the bridge of dentin overlying the pulp. This would be accompanied by a bending of the buccal and lingual cavity walls which would cause a separation of tooth and restoration under the action of occlusal loading.

SUMMARY AND CONCLUSIONS

The resistance form of a disto-occlusal amalgam restoration in a primary mandibular first molar was investigated. The significant stresses were analyzed in both the tooth and restoration and the following conclusions were drawn.

1. The failure of this restoration at the isthmus is due to the presence of tensile stresses in this region.

2. The tensile stress is maximum at the occlusal surface of the restoration, thereby specifying this region as one from which fracture may be easily propagated.

3. Any surface discontinuities in this critical region, such as carved developmental grooves, roughened surface texture, or inadvertent scratches or nicks, will produce deleterious stress concentrations.

4. The tensile stress at the occlusal surface increases in magnitude as the distance from the axial wall is increased. The isthmus should, therefore, be placed as close to the axial wall as possible.

5. Of the 4 designs tested, the tensile stress in the restoration was least for the sloping axial wall in combination with the flat pulpal wall.

6. From the standpoint of tooth structure, increasing the width of the isthmus, as well as increasing the depth, both result in an increase in the internal stress within the tooth, thereby lowering its resistance to structural deformation and failure.

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THE AVAILABILITY OF CERTAIN STREPTOCOCCAL GROWTH FACTORS IN HUMAN SALIVA

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THE streptococci, which are generally recognized as being among the most numerous organisms to be found in the mouth, have been investigated from a nutritional standpoint by various authors. Smiley, Niven, and Sherman,[1] for example, pointed out that *Streptococcus salivarius* required 5 members of the "B" group of vitamins for growth in a synthetic medium and Niven and Sherman[2] studied *Streptococcus faecalis* in a similar manner. Nevin[3] confirmed their results insofar as vitamin requirements are concerned, using streptococci freshly isolated from the mouth. It seemed profitable, therefore, to determine the availability for streptococcal growth of certain factors in saliva relatable in activity to the specific B vitamins, niacin, pantothenic acid, riboflavin, and biotin.

METHODS AND MATERIALS

The organisms used as assay tools were *Streptococcus salivarius* A.T.C.C. 9756 and *Streptococcus faecalis* A.T.C.C. 828. The latter strain was used for assaying biotin since its growth response was more nearly linear in this system. Inocula were prepared by repeated cultivation in a basal medium supplemented with riboflavin, pantothenic acid, niacin, biotin, thiamin, pyridoxamine, and folic acid. The factor to be assayed was supplied to the starter culture in the smallest amount which would permit maximal growth.

The basal medium used was similar to that described by Smiley, Niven, and Sherman,[1] except that a mixture containing 1 mg. per milliliter of each of 15 amino acids was added. The amino acid supplement was substituted satisfactorily for a charcoal adsorbed yeast extract found to be necessary by Niven and Smiley[4] for the use of *Str. salivarius* in a thiamin assay. Since the addition of filter sterilized thiamin to the medium, after autoclaving, yielded more satisfactory growth responses than those obtained when this vitamin was added to the medium before autoclaving, a 0.1 ml. volume of a standard thiamin solution was always introduced into each tube of assay medium as a sterile additive. After addition of the vitamins, other than thiamin and the factor to be assayed, the media were adjusted to pH 6.6 and diluted to 80 per cent of final volume. They were then dispensed, in 4 ml. amounts, into Pyrex glass

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culture tubes suitable for use in the Klett-Summerson colorimeter. The addition of 1 ml. aliquots of appropriate reference solutions of the limiting factor, in triplicate, provided quantitative standards. Sterilization was accomplished by autoclaving at 121° C. for 15 minutes.

TABLE I

COMPOSITION OF THE ASSAY MEDIUM

Acid hydrolyzed "vitamin free" casein	0.5%
Glucose	0.5%
K_2HPO_4	0.4%
Uracil	0.5 mg. %
Sodium thioglycollate	10.0 mg. %
Amino acid mixture* (mg./ml. of each of 15 amino acids)	4 ml./100 ml.
Salts†	
Riboflavin	100 μg %
Niacin	100 μg %
Ca. pantothenate	100 μg %
Pyridoxamine	100 μg %
Folic acid	100 μg %
Thiamin HCl	10 μg %
Biotin	100 mμg %

*L cystine. L cysteine HCl, L glutamic acid, DL methionine. L arginine HCl. L asparagine, DL alphaalanine, DL isoleucine, DL leucine, L aspartic, L lysine HCl, DL phenylalanine, DL serine, L tyrosine, glycine.

†$MgSO_4 \cdot 7H_2O$, 80.0 mg.; $FeSO_4 \cdot 7H_2O$, 0.4 mg.; $MnCl_2$, 0.12 mg.; NaCl, 2.0 Gm. per 1,000 ml. complete medium.

One drop of the inoculum, an 18- to 24-hour fluid culture, was added to each assay tube by means of a sterile 10 ml. serological pipette. Initial and final Klett readings were made on each assay tube using the 660 mμ (red) filter. A water bath at 35° C. was used for incubation over a 24-hour period. Standard curves were constructed showing growth response to riboflavin, pantothenic acid, niacin, and biotin. These were done concurrently with each series of samples. When aseptic additions were made, the additives were checked for sterility routinely.

One hundred nonstimulated saliva specimens were collected from dental students and from various members of the Department of Bacteriology. Collections were made at least 2 hours after the most recent ingestion of food. Aliquots of 5 individual specimens were pooled. Each pool was then divided into thirds in order to compare extraction methods (aqueous, acid, and heat).

Aqueous extracts were prepared by the addition of equal volumes of water, followed by vigorous shaking for 1 minute. Gross particulates were removed by centrifugation at 2,500 r.p.m. The supernatants were then sterilized by passage through presterilized U.F. sintered glass filters.

Acid extracts were prepared by the addition of 1 ml. of normal HCl to 5 ml. of whole saliva. The mixtures were incubated at 30° C. overnight, then adjusted to pH 6.6 with normal NaOH and diluted to total volumes of 10 ml. Centrifugation and sterilization were as described for the aqueous extracts.

Heat extraction and simultaneous sterilization were accomplished by the addition of an equal volume of distilled water to whole saliva, and autoclaving at 121° C. for 15 minutes. Particulates were removed by centrifugation

and the clear supernatant studied. In all cases, 1 ml. amounts of the sterile extracts were added aseptically to each of 4 assay media. All assays were carried out in duplicate.

RESULTS

Using the described methods, it became apparent that the means of extraction altered significantly the amounts of growth factors available in saliva. Aqueous extraction yielded the lowest amounts of activity, whereas heat extraction yielded the greatest amounts of activity of the methods tested (Table II).

TABLE II

THE EFFECT OF THE EXTRACTION METHOD ON GROWTH FACTOR ACTIVITY IN 20 POOLED SPECIMENS OF SALIVA

ACTIVITY PER ML. WHOLE SALIVA	EXTRACTED WITH		
	WATER	ACID	HEAT
Pantothenate (μg)	0.033	0.038	0.071
Niacin (μg)	0.016	0.120	0.127
Riboflavin (μg)	0.027	0.068	0.142
Biotin (mμg)	0.025	0.059	0.281

The tabulated figures indicate that heat extraction approximately doubled the availability of pantothenate, as compared to aqueous extraction. Similarly, niacin activity increased 8 times; riboflavin 5 times; and biotin 11 times. Acid extraction did not appreciably increase the availability of pantothenic acid over aqueous extraction, whereas riboflavin and biotin activities were doubled. Niacin was, as in the case of heat extraction, increased about eightfold.

Since it was of interest to know something of the origin of growth factors in saliva, experiments were designed to examine the role of soluble food residues after a meal. Therefore, salivary specimens were collected from 12 selected individuals just before, and 15 minutes after, eating a meal of ham, bread, margarine, lettuce, milk, and doughnuts. No limitation was placed on the amount eaten. The saliva samples were extracted aqueously. The means and the range of values of pantothenic acid, riboflavin, and biotin activities

TABLE III

THE EFFECT OF SOLUBLE FOOD RESIDUES ON THE AVAILABILITY OF BACTERIAL GROWTH FACTORS IN AQUEOUS EXTRACTS OF SALIVA

ACTIVITY PER ML. WHOLE SALIVA		BEFORE MEAL	AFTER MEAL	PER CENT DIFFERENCE
Pantothenate (μg)	Mean	0.039	0.035	-10.3
	Range	0.031-0.05	0.029-0.05	
Riboflavin (μg)	Mean	0.058	0.057	-0.17
	Range	0.042-0.075	0.042-0.083	
Biotin (mμg)	Mean	0.015	0.042	+180.0
	Range	0.004-0.03	0.014-0.10	

are summarized in Table III. The levels of pantothenate and riboflavin activity were not appreciably changed, whereas biotin activity was considerably increased. More recent experiments indicate that niacin activity also remains essentially unchanged, while biotin levels may be only moderately increased.

DISCUSSION

The presence of B vitamins or vitamin-like compounds in saliva has been reported on several occasions. Weisberger,[5] measured activity relatable to niacin and pantothenic acid. His purpose was the evaluation of saliva as a medium for studying acid production by a strain of Lactobacillus. Acid hydrolysis of the salivary specimen did not increase pantothenic acid activity. This observation is in agreement with present results. The effect of acid hydrolysis on niacin activity was not described. Pantothenic acid concentrations, ranging from 0.012 μg to 0.19 μg per milliliter of whole saliva, were reported by Kniesner, Mann, and Spies.[6] A mean of 0.1 μg per milliliter was calculated from their data. The niacin content of whole saliva, as determined by Dreizen, Reed, and Spies,[7] ranged from 0.0234 μg to 0.409 μg per milliliter. The mean of these data was calculated to be 0.1 μg per milliliter also.

Glavind, Granados, Hansen, Schilling, Kruse, and Dam[8] assayed pooled saliva for most of the known vitamins. Those of interest in this report were present as follows: pantothenic acid, 0.08 μg; niacin, 0.03 μg; riboflavin, 0.05 μg; biotin, 0.0008 μg per milliliter of whole saliva.

The preparation of salivary samples for assay was not described in detail by the cited investigators. It has been assumed that their samples were autoclaved in the assay medium. Such treatment would conform to the method originally described by Snell and Wright,[9] which was used for niacin determinations, and that of Pennington, Snell, and Williams,[10] which was used to measure pantothenic acid levels.

The activities demonstrable in heat-treated saliva reported herein approximate those previously published. Such levels of activity probably do not occur naturally in the mouth. The lower levels, demonstrable by aqueous extraction of saliva, appear more nearly representative of those which are likely to occur.

The strongly acidogenic character of such organisms as the streptococci, if given sufficient amounts of fermentable carbohydrates, could conceivably be active in bringing about partial hydrolyses of saliva in localized areas. Thus, intermediate levels of vitamin activity, as evidenced by acid extraction of saliva, might in some instances occur under natural circumstances. The increased niacin activity in relation to increased dental caries, reported by Dreizen, Reed, and Spies,[7] previously cited, might be so explained.

The origin of the growth factor activity described in saliva is still obscure. It is possible that the bound forms, those recovered by more vigorous extraction methods, were derived in some part from indigenous microorganisms and cellular debris. The "free" growth factors were probably derived from the same sources as a result of natural degrading processes. While it is also possible that soluble food residues in the mouth may furnish such compounds directly, assays of aqueous extracts of saliva collected before and after a standard meal revealed an increase in biotin activity only. It is not known whether this was due to an actual increase in biotin or to substances such as fatty acids which may substitute for biotin in the metabolism of *Str. faecalis*.

SUMMARY

Using standard microbiological assay methods, adapted for the utilization of streptococci which may occur in the oral cavity, significant amounts of growth factor activity relatable to the 4 vitamins, riboflavin, niacin, pantothenic acid, and biotin, were demonstrable in nonstimulated human saliva.

In addition, it has been shown that by increasingly drastic extraction methods, increasingly larger amounts of activity are usually evidenced.

Finally, the levels of activity of free pantothenic acid and riboflavin were not increased after the ingestion of a standard meal, whereas the level of biotin activity was increased.

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THE TEETH OF ALLIGATOR MISSISSIPPIENSIS DAUD

II. Development of Dentin

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IN fish and Amphibia, the organic foundation of the entire tooth is a product of the dental papilla, the outer part of it being more highly mineralized and forming the mesodermal enamel.[3, 4] In Reptilia, for the first time in phylogenesis, an ectodermal enamel covering of the tooth occurs, whereas the function of the dental papilla is confined to the production of the dentin proper.[3]

MATERIAL AND METHODS

The animals investigated were 36 cm. long. Immediately after death, the jaws were dissected out, skinned, and divided into pieces 2 to 3 mm. in length. The specimens were fixed in Baker's[1] formol-calcium, Bouin or Zenker formol. The material was decalcified in 5 per cent trichloracetic acid, embedded in paraffin or double-embedded in celloidin-paraffin,[7] and cut in series. The thickness of the sections varied from 4 to 7 microns. Staining methods employed were hematoxylin-eosin, Masson's trichrome with light green, and Heidenhain's azan. For demonstration of the reticular or precollagenous fibers, Wilder's silver impregnation method was used.

OBSERVATIONS

After the inner epithelial cells around the tip of the tooth germ have elongated into columnar cells, differentiation of the cells in the tip of the dental papilla into dentinoblasts commences.[5] Their nuclei increase in size and are situated at varying distances from the outer border of the papilla (Fig. 1).

At a stage where some early dentin is present, the dentinoblastic cytoplasm has increased considerably (Fig. 2). In the smaller cells the nucleus is centrally positioned (Fig. 2, *c*), whereas the nucleus comes to lie further from the dentin surface as cell size increases (Fig. 2, *d*). The cytoplasm of all dentinoblasts extends to the inner dentinal border, which, in these young cells, has a dense granular or striated appearance (Figs. 2 and 3).

As dentin development progresses, the dentinoblasts in the tip of the dental papilla increase in number (Fig. 4). Adjacent to the dentin is a broad band of cytoplasm, with the nuclei mostly situated at the papillary side of the

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dentinoblast layer. The cytoplasmic structure is less dense, with fibrous formed elements running principally longitudinally in respect to the axis of the tooth germ, but with smaller fibrils branching from the longitudinal fibers and extending rather irregularly. The fibers accept the cytoplasmic dyes. Cell borders between the individual dentinoblasts are now absent.

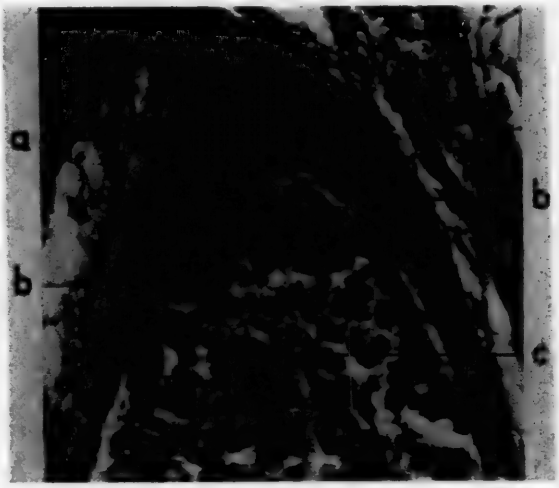

Fig. 1.—Tip of tooth germ before dentin formation. *a*, Tooth germ's inner epithelium; *b*, membrana terminans; *c*, dental papilla. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×600.)

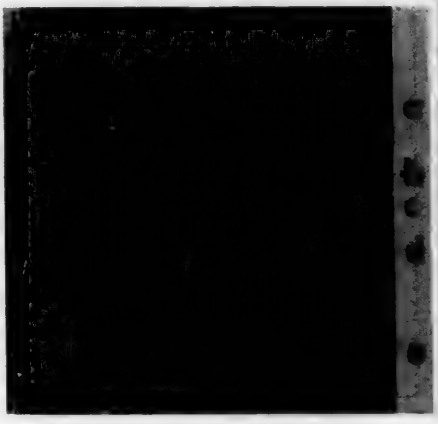

Fig. 2.

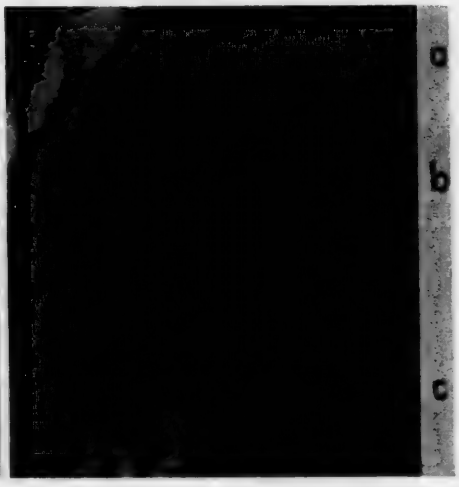

Fig. 3.

Fig. 2.—Tip of tooth germ with beginning of dentin formation. *a*, Tooth germ's inner epithelium; *b*, dentin; *c*, dentinoblast near dentin; *d*, dentinoblast with nucleus remote from dentin and cytoplasm extending to dentin; *e*, dental papilla. (Masson's trichrome with light green; Orig. mag. ×600.)

Fig. 3.—Tip of tooth germ with dentin formation in progress. *a*, Tooth germ's inner epithelium; *b*, dentin; *c*, dental papilla. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×600.)

The broad dentinoblast layer at the tip of the papilla becomes narrower along the sides where the dentin is yet of small width (Fig. 5). In these younger sites of dentin formation, the dentinoblasts are columnar cells of about equal height with their nuclei situated at varying levels.

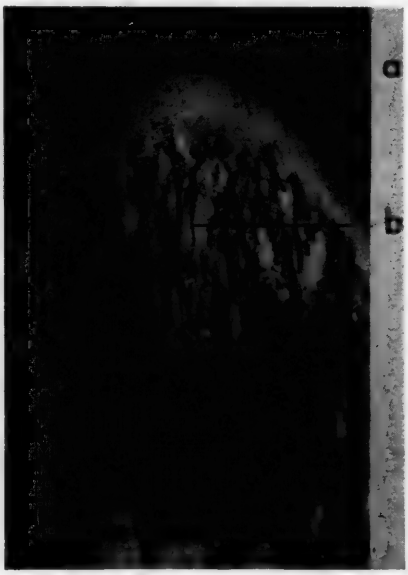

Fig. 4.—Dentin forming zone. *a*, Dentin; *b*, dentinoblasts with fibroglia fibers. (Masson's trichrome with light green; Orig. mag. ×360.)

Fig. 5.

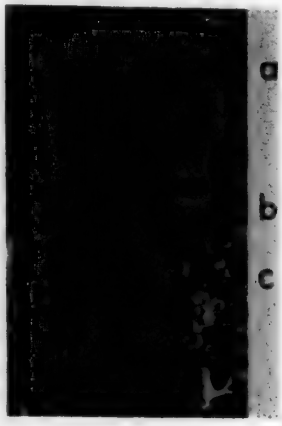

Fig. 6.

Fig. 5.—Dentin forming zone. *a*, Tooth germ's inner epithelium; *b*, dentin; *c*, dentinoblasts; *d*, dental papilla with argyrophilic fibers. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×360.)

Fig. 6.—Base of tooth germ. *a*, Tooth germ's inner epithelium; *b*, membrana terminans; *c*, dental papilla with argyrophilic fibers. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×600.)

At the base of the dental papilla, where dentin has not yet formed, the undifferentiated cell types extend nearly to the inner epithelium of the tooth germ, leaving a narrow cell-free zone adjacent to the epithelium (Fig. 6).

In silver stained sections, certain elements of the dental papilla are argyrophilic. Before dentin forms, a fine silver stained line is seen just inside the tooth germ's inner epithelium (Fig. 1, *b*). This is the membrana terminans, a structure which is especially distinct in tooth germs of Selachii. It has been demonstrated to be built up of precollagenous connective tissue fibers.[4]

During dentin development the papilla has a dense network of argyrophilic fibers (Figs. 5, *d* and 7, *c*). At its base, where no dentin has as yet formed, the fiber plexus extends to the inner epithelium, where it is confluent with membrana terminans (Fig. 6).

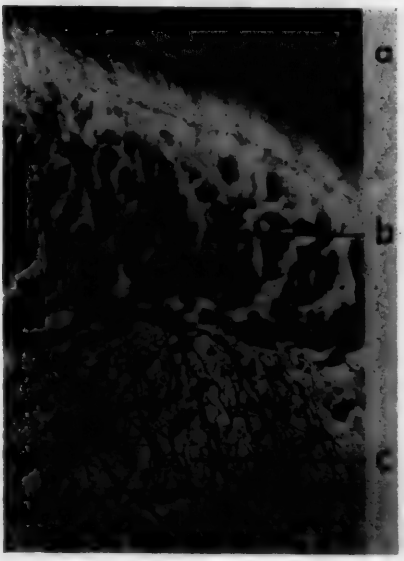

Fig. 7.—Dentin forming zone. *a*, Dentin; *b*, dentinoblasts; *c*, dental papilla with argyrophilic fibers. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×360.)

At sites where the dentinoblasts are morphologically differentiated and dentin formation has begun, argyrophilic fibers were never observed in the dentinoblastic layer (Figs. 5, *c* and 7, *b*). The silver stained fibers could be traced to the inner border of the dentinoblasts, but could never be demonstrated to enter into or to transverse the dentinoblastic layer.

The first dentin which is formed in the tip of the dental papilla (Fig. 2, *b*) is built up of irregularly oriented fibers, but those following the peripheral contour predominate. In sections stained with Masson's trichrome with light green, the dentin layer is bright green in color, similar to staining of collagenous fibers. There is an abrupt transition in color from this green layer to the red-colored cytoplasm of the dentinoblasts.

A somewhat wider dentin band (Fig. 3) also takes a distinct collagenous stain, but the change in color to the cytoplasmic staining of the dentinoblasts is not so abrupt as in the earliest stages of dentin formation.

The precise picture of the zone of forming dentin is better observed where development has progressed somewhat further. The innermost layer of dentin

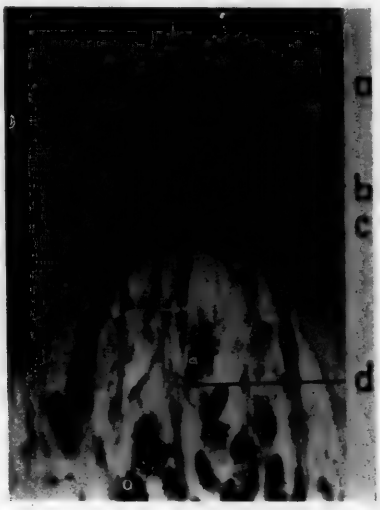

Fig. 8.

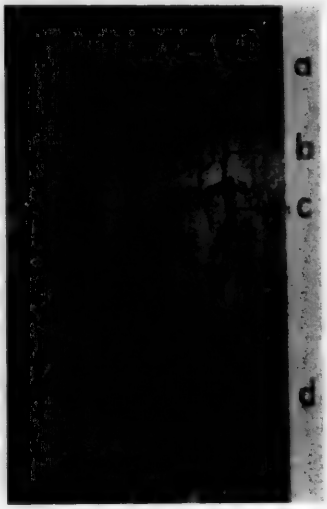

Fig. 9.

Fig. 8.—Dentin forming zone. *a*, Dentin; *b*, calcified dentinal stripe; *c*, dentinal tubule; *d*, dentinoblasts. (Masson's trichrome with light green; Orig. mag. ×540.)

Fig. 9.—Dentin forming zone. *a*, Dentin; *b*, calcified dentinal stripe; *c*, fibroglia fiber; *d*, dentinoblasts. (Masson's trichrome with light green; Orig. mag. ×600.)

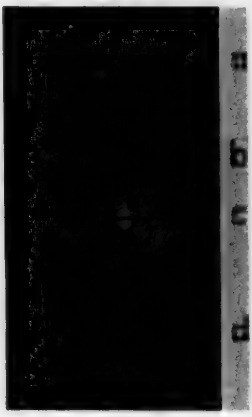

Fig. 10.

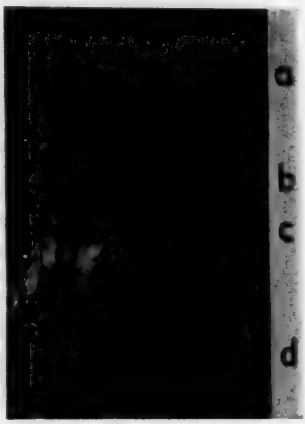

Fig. 11.

Fig. 10.—Dentin forming zone. *a*, Dentin; *b*, calcified dentinal stripe; *c*, fibroglia fiber; *d*, dentinoblasts. (Masson's trichrome with light green; Orig. mag. ×600.)

Fig. 11.—Dentin forming zone. *a*, Dentin; *b*, calcified dentinal stripe; *c*, fibroglia fiber; *d*, dentinoblasts. (Masson's trichrome with light green; Orig. mag. ×600.)

stains fainter than the rest of the dentin (Fig. 8, *b* and *c*), probably due to a lesser degree of calcification. This is the predentin and in sections is composed of darker, parallel stripes representing the calcified part of the dentin (Fig. 8, *b*), and lighter stripes between, which are the openings of the dentinal tubules into the dental papilla (Fig. 8, *c*). The calcified dentinal stripes are composed of fibers which are twisted in bundles and stain like collagenous fibers. They are broadest in the peripheral part and taper to a point at the margin of the dental papilla.

The dentinal tubules are of greatest width at the opening to the dental papilla (Fig. 8, *c*) and progressively decrease in diameter as they penetrate into the dentin. At the widest part of the tubules they are seen to take a distinct cytoplasmic stain and there is no change in structure from the cytoplasm of the dentinoblasts to the content of the tubules.

The intracellular fibers of the dentinoblasts are often seen to be continuous with the fibrous calcified dentinal stripes (Figs. 9, 10, and 11). The cytoplasmic stain of the intracellular fibers is gradually altered into the collagenous stain of the dentinal fibers.

DISCUSSION

The outermost layer of dentin is formed at a site where the membrana terminans (Fig. 1, *b*) and the argyrophilic fibers of the dental papilla (Fig. 6, *b* and *c*) were seen to be established in younger developing teeth. It is reasonable to infer that these argyrophilic, precollagenous fibers are transformed to collagenous fibers and, as such, are incorporated into the dentin.

After any initial dentin is present, the argyrophilic fibers of the papilla always terminate at the inner border of the dentinoblastic layer and are never observed within this cell layer (Figs. 5 and 7). This means that the argyrophilic fibers take no part in the formation of dentin after the outermost layer of dentin has been laid down.

The intracellular fibers of the dentinoblasts (Fig. 4) are probably identical with the fibers in the cytoplasm of the cells of the papilla previously described as forming the organic foundation of the mesodermal enamel in *Squalus acanthias*,[4] and which were called fibroglia fibers according to Mallory's[6] terminology. Mallory[6] found that the fibroglia fibers took a distinct cytoplasmic stain; that they are numerous and predominant when the connective tissue cells are numerous, active, and proliferating, whereas they are difficult to demonstrate when the cells are few in number or in an inactive state. These characteristics coincide with the present observations of the dentinoblasts in *Alligator mississippiensis*. The lack of cell borders suggests that the dentinoblasts form a syncytium. That the fibroglia fibers often extend continuously over a wider field than can be accepted as a single cell also points to the syncytial character of the cell layer.

The calcified dentinal stripes (Fig. 8, *b*), especially in the predentin, are seen to be composed of twisted fibers. They stain similarly to collagenous

fibers and probably are the collagenous fibers of the dentin. As calcification proceeds, the individual fibers are not so readily observed.

A direct continuity from fibroglia fibers of the dentinoblasts to a bundle of collagenous fibers of the predentin (Figs. 9, 10, and 11) and a gradual change in color in this transition from the cytoplasmic stain of the fibroglia fiber to the collagenous stain of the dentinal fibers indicates that the fibroglia fibers are transformed to collagenous fibers and thus give rise to the collagenous fibers of the dentin.

A similar transition from fibroglia fibers to collagenous fibers has previously been described in the formation of mesodermal enamel in *Sq. acanthias.*[4] Here the cell nuclei of the papilla tip withdrew toward the papilla base and the cytoplasm of the cells collected in the papilla tip. During the further development, the stainability of the fibroglia fibers in the cytoplasm changed from a cytoplasmic stain to a collagenous stain. Kerr[2] observed the same change in fiber color in the course of enamel development in *Sq. acanthias.*

Since the dentinal tubules contain a substance similar in structure and staining characteristics (Fig. 8, *c*) to the cytoplasm of the dentinoblasts, it appears that the tubules include projections of cellular cytoplasm.

According to the present observations, the formation of dentin inside of the initial outer dentin layer in *A. mississippiensis* is effected through a transformation of the dentinoblasts. The fibroglia fibers are converted into collagenous fibers and give rise to the fiber system of the dentin. The rest of the cytoplasm is retained within the tubules between the calcified dentinal stripes containing the collagenous fibers.

The calcified dentinal stripes start pointed and become gradually broader toward the periphery (Fig. 8, *b*), whereas each dentinal tubule has a wide opening toward the dental papilla and narrows considerably as it extends more deeply into the dentin (Fig. 8, *c*). This indicates that the calcified dentinal stripes grow at the expense of the dentinal tubules. Whether this new formation of collagenous fibers is effected by growth of the fibers already in existence or by transformation of smaller fibroglia fibers in the dentinal tubules cannot be decided on the basis of the present observations.

SUMMARY

The organic foundation of the outermost dentin layer in *Alligator mississippiensis* Daud is formed with participation of precollagenous argyrophilic fibers which are transformed to collagenous fibers. The rest of the organic dentinal matrix originates by transformation of the cytoplasm of the dentinoblasts, the fibroglia fibers of the cytoplasm being converted to collagenous fibers and giving rise to the fiber system of the dentin, the rest of the cytoplasm filling the dentinal tubules.

ADDENDUM

After the completion of this paper, an extensive work was published by W. Warwick James: A Further Study of Dentine, Trans. zool. Soc. Lond. 29: Part 1, August, 1957.

James denies the presence of cytoplasmic processes in the dentinal tubules, these being permeated by tissue fluid. The odontoblasts, in James' opinion, give rise to the collagenous fibers and mucopolysaccharides form the dentin matrix.

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THE TEETH OF ALLIGATOR MISSISSIPPIENSIS DAUD

III. Development of Enamel

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MOST teeth have an outer stratum where calcification advances to the point of high acid solubility.[1] The organic foundation of this layer in fish and amphibian teeth is a product of the mesodermal dental papilla, and has thus been termed mesodermal enamel.[1, 2] Increase in size of the columnar epithelial cells of the tooth germ during calcification of the mesodermal enamel is indicative of cellular activity related to its calcification.[3]

At the next step of phylogenetic development of the enamel, the columnar epithelium of the tooth germ takes over the formation of the organic foundation as well as its calcification. This ectodermal enamel is seen for the first time in reptilian teeth. A precise description of the elaboration of such early ectodermal enamel has not previously been given in the literature.

MATERIAL AND METHODS

The material and its treatment in preparing the jaws, fixation, decalcification, embedding, sectioning, and staining was the same as that employed in the study of the dentin development.[5]

As most of the organic enamel matrix disappears in routine decalcification, special methods were tried to maintain the matrix in the sections.

Some dental follicles were dissected out before fixation with the aid of a binocular dissecting microscope. After fixation and embedding in a very hard nitrocellulose block,[6] the tooth buds were cut undecalcified. This method was successful only in the first stages of enamel development owing to the rapid hardening of the enamel matrix.

Other tooth buds were infiltrated with 10 per cent nitrocellulose after fixation, and decalcified in 10 per cent nitrocellulose with 5 per cent formic acid. After neutralization in 4 changes 10 per cent nitrocellulose, they were infiltrated in 25 per cent and 50 per cent nitrocellulose and embedded in 50 per cent nitrocellulose solution.

OBSERVATIONS

At the earliest developmental stages of the tooth germ, the inner epithelial cells are cuboidal in shape. The beginning differentiation of these cells

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Fig. 1.

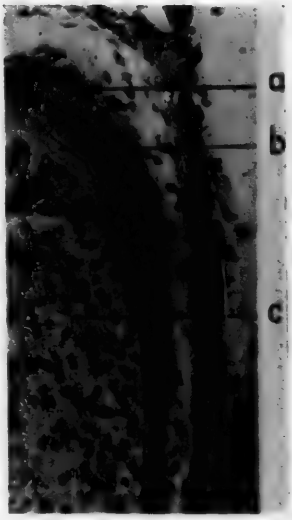

Fig. 2.

Fig. 1.—Tip of tooth germ. *a*, Beginning differentiation of ameloblasts; *b*, dental papilla. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×360.)

Fig. 2.—Tip of tooth germ. *a*, Differentiation of ameloblasts; *b*, dentin; *c*, dental papilla. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×360.)

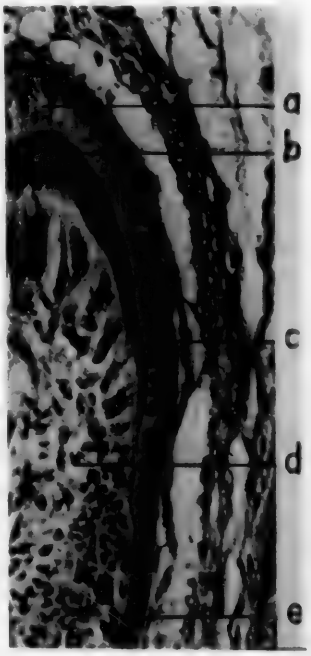

Fig. 3.

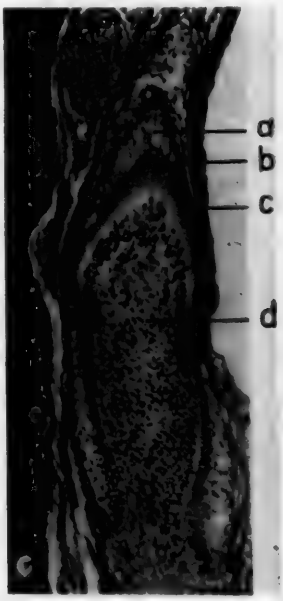

Fig. 4.

Fig. 3.—Tip of tooth germ. *a*, Ameloblasts with peripheral nuclei; *b*, dentin; *c*, differentiation of ameloblasts; *d*, dental papilla; *e*, undifferentiated inner epithelium. (Wilder's silver impr., Masson's trichrome with light green; Orig. mag. ×360.)

Fig. 4.—Longitudinal section of tooth germ. *a*, Ameloblasts; *b*, enamel matrix; *c*, dentin; *d*, dental papilla. (Masson's trichrome with light green; Orig. mag. ×120.)

into ameloblasts occurs at the tip of the tooth germ where the inner epithelium broadens (Fig. 1, *a*). The cell nuclei enlarge, their form becomes more irregular, and they are situated at varying distances from the dental papilla.

The next step in the development of the ameloblasts is a growth of the cytoplasm of the inner epithelial cells (Fig. 2, *a*). The nuclei are still scattered at different levels of the epithelial layer.

The width of the tooth germ's inner epithelium increases as the cytoplasm of the cells enlarge (Fig. 3, *a*). The cytoplasm has a granulated appearance at this early stage. The nuclei lie in an approximately even line at the periphery of the cells which have attained a typical columnar shape.

Along the wall of the tooth germ, progressive stages in the development of the ameloblasts can be traced in the same section. At a level where dentin development has well started the cells are somewhat elongated with the nuclei at different levels in the cells (Fig. 3, *c*). Nearer the base of the tooth germ, the outer epithelial cells have a cuboidal shape with centrally situated nuclei (Fig. 3, *e*).

Where deposition of enamel matrix is present in the tip of the tooth germ, the ameloblasts have still greater length (Fig. 4, *a*). The earlier granulated appearance of the cytoplasm has changed, such that the cell bodies more frequently are built up of fibers which dominantly extend longitudinally to the cell axis. In the methods employed, the fibers take a distinct cytoplasmic stain.

In the subsequent stages of tooth development, where enamel formation occurs along the sides of the tooth germ, the matrix often appears before the ameloblasts have attained their ultimate size (Fig. 5). The increase of cytoplasm continues as the deposition of enamel matrix advances, and the nuclei retain their position in the outer ends of the ameloblasts. The ameloblasts along the sides of the tooth germ, however, seldom become as large as do the inner epithelial cells around the tip. The striated or fibrous appearance of the active ameloblasts is common for the whole tooth germ.

The ameloblasts maintain their size and appearance during the deposition of enamel matrix and as calcification proceeds. Even when the matrix is calcified so heavily that it is dissolved during decalcification (Fig. 6, *b*), no alterations are seen in the ameloblasts.

It is only at the later developmental stages, after most of the enamel is deposited, that the ameloblastic layer shrinks to a narrow stripe (Fig. 7, *a*), and the individual ameloblasts lose their structure and assume a pycnotic character.

The first enamel matrix which is laid down in the tip of the tooth has a faint striation (Fig. 8, *b*). The striae are indicative of a fibrous structure. The orientation of the fibers varies. In some places they extend perpendicularly to the direction of the matrix layer (Fig. 8, *b*), while in others they are parallel to this layer (Fig. 8, *c*). The intracellular fibers of the ameloblasts are in some instances seen to be continuous with the fibers of the enamel matrix, with only a change in color at the transition between cell and matrix.

At the beginning of enamel matrix formation down the side of the tooth germ, a distinct striation is often seen in the matrix (Fig. 9, *b*). The fibers

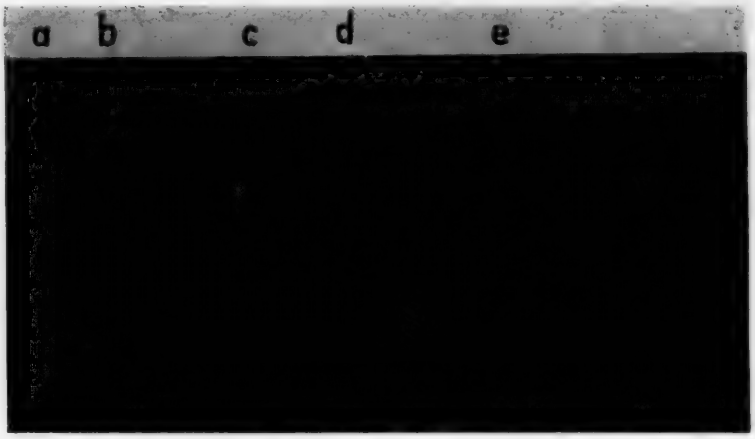

Fig. 5.—Detail of longitudinal section of tooth germ. *a*, Dentin; *b*, enamel matrix; *c*, ameloblasts with tonofibrils; *d*, transition tonofibril enamel matrix fiber; *e*, dental papilla. (Azan; Orig. mag. ×800.)

Fig. 6.

Fig. 7.

Fig. 6.—Tip of tooth germ. *a*, Ameloblasts; *b*, enamel dissolved in decalcification; *c*, dentin; *d*, enamel matrix. (Masson's trichrome with light green; Orig. mag. ×360.)

Fig. 7.—Detail of longitudinal section of tooth germ. *a*, Ameloblasts after completed function; *b*, dentin. (Masson's trichrome with light green; Orig. mag. ×360.)

here always extend perpendicularly to the longitudinal axis of the tooth. In some places a direct transition is seen between the intracellular fibers of the ameloblasts and the fibers of the enamel matrix (Figs. 5, *d* and 10, *b*).

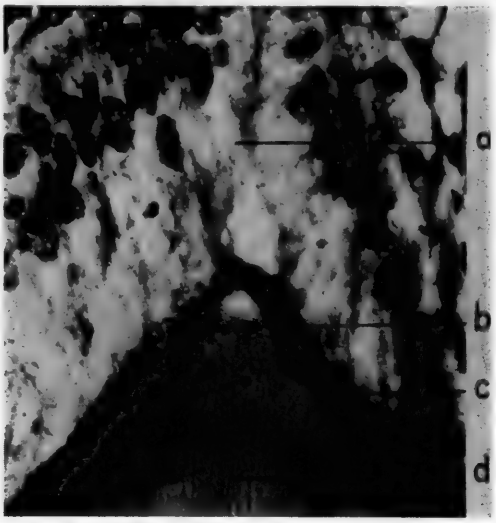

Fig. 8.—Tip of tooth germ. *a*, Ameloblasts; *b*, enamel matrix with perpendicular fibers; *c*, enamel matrix with longitudinal fibers; *d*, dentin. (Masson's trichrome with light green; Orig. mag. ×800.)

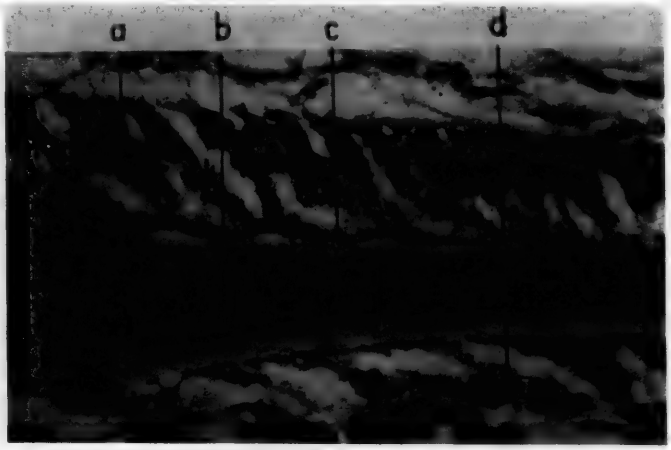

Fig. 9.—Detail of longitudinal section of tooth germ. *a*, Ameloblasts; *b*, enamel matrix with perpendicular fibers; *c*, dentin; *d*, dental papilla. (Masson's trichrome with light green; Orig. mag. ×800.)

The youngest enamel matrix in later developmental stages is ordinarily very coloraffin.[1] The intense staining masks most of the structure (Figs. 11, *b* and 12, *b*), but in some areas the characteristics can be faintly outlined (Fig. 11, *b*). As calcification proceeds the staining is less intense, and the fibrous nature of the matrix becomes obvious (Figs. 11, *c* and 12, *c*).

Calcification of the enamel matrix proceeds very rapidly. In advanced stages of dental development only the last deposited matrix is preserved in the sections (Fig. 13, *d*). In the next older part of the tooth only the matrix

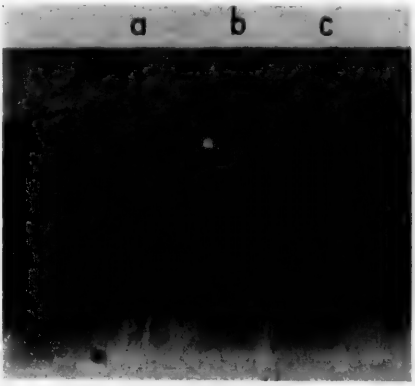

Fig. 10.

Fig. 11.

Fig. 10.—Detail of longitudinal section of tooth germ. *a*, Ameloblasts with tonofibrils; *b*, transition tonofibril enamel matrix fiber; *c*, enamel matrix. (Azan; Orig. mag. ×800.)

Fig. 11.—Detail of longitudinal section of tooth germ. *a*, Ameloblasts; *b*, coloraffin enamel matrix; *c*, fibrous enamel matrix. (Azan; Orig. mag. ×800.)

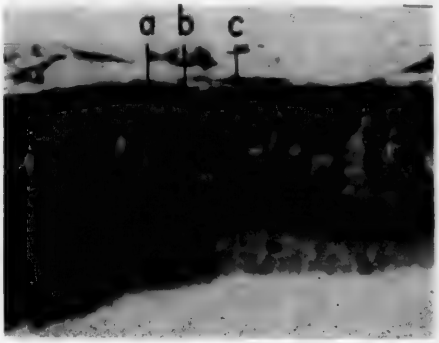

Fig. 12.

Fig. 13.

Fig. 12.—Detail of longitudinal section of tooth germ. *a*, Ameloblasts; *b*, coloraffin enamel matrix; *c*, fibrous enamel matrix. (Masson's trichrome with light green; Orig. mag. ×800.)

Fig. 13.—Longitudinal section of tooth germ. *a*, Ameloblasts; *b*, dentin; *c*, enamel dissolved in decalcification; *d*, enamel matrix; *e*, dental papilla. (Masson's trichrome with light green; Orig. mag. ×45.)

nearest the ameloblasts is preserved, whereas the central part of it was so heavily calcified that it was dissolved by the decalcifying acid. In the rest of the tooth the whole enamel layer has disappeared during decalcification (Fig. 13, *c*).

DISCUSSION

The intracellular fibers of the ameloblasts are probably the tonofibrils which have previously been described in epithelial cells. They are likely of the same nature as the fibroglia fibers of the fibroblasts which take an important part in the elaboration of the mesodermal enamel in *Squalus acanthias* and some osseous fish,[2] and in the development of dentin in *Alligator mississippiensis*.[5]

Tonofibrils are not seen in the early stages of the ripening of the ameloblasts (Fig. 3, *a*), but they are often observed during enamel matrix formation (Figs. 4, *a*, 5, *c* and 10, *a*). This may be indicative of their taking an active part in enamel development. The further observation of continuation between certain tonofibrils and fibers of the enamel matrix may indicate that the tonofibrils give rise to the fibers of the enamel (Figs. 5, *d*, 8, *b* and 10, *b*).

Since some of the main elements of the ameloblasts can be followed without interruption from the ameloblasts to the enamel matrix and, as we often find a transition from the ameloblasts to the enamel matrix without any borderline or intermediate layer (Fig. 10), there is basis for an opinion that the enamel matrix is formed by growth of the ameloblasts simultaneously with a transformation. This latter is evidenced by a change in stainability.

SUMMARY

The cytoplasm of the functioning ameloblasts in *Alligator mississippiensis* Daud contains tonofibrils which are continuous with fibers in the enamel matrix running perpendicularly to the longitudinal direction of the tooth.

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THE REACTION OF METAL OXIDES WITH *o*-ETHOXYBENZOIC ACID AND OTHER CHELATING AGENTS

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ZINC oxide-eugenol mixtures form a hard, coherent mass that has been useful in a number of dental applications. The hardened mass consists of zinc oxide embedded in a matrix of a zinc eugenolate chelate with the empirical formula $(C_{10}H_{11}O_2)_2Zn$.[1] Mixtures of zinc oxide with ortho substituted phenols such as *iso*eugenol, guaiacol, and 4-methylguaiacol also harden. As would be expected from the reaction mechanism, phenols that do not contain ortho substituents or that contain substituents that are incapable of chelation do not undergo this reaction.

The presently available commercial zinc oxide-eugenol mixtures harden rapidly only in the presence of accelerators. The products have low compressive strength. The materials inhibit the polymerization of acrylic monomers. Hence, acrylic resins will not polymerize in direct contact with these materials. It therefore appeared of interest to study the reaction of zinc oxide with a number of commercially available chelating agents. A large portion of this report deals with the products obtained from mixtures of zinc oxide with *o*-ethoxybenzoic acid (EBA) because they appeared to be the most promising.

EXPERIMENTAL PROCEDURES

Materials.—Zinc oxide reagent grade was used in most experiments. Over 50 per cent of the powder passed through a No. 70 sieve but was retained by the No. 100 sieve. An experimental zinc oxide (Merck Hyperfine) having a particle size of about 0.02 micron in diameter and containing approximately 5 per cent of water was also used. Analysis of the sample showed the presence of 1.2 per cent of ammonia.

Other materials used were: zinc oxides XX-078, Kadox-72, Kadox-15 and No. 513† (particle size of these materials is given in Table VI); micronized U. S. P. zinc oxide‡; tetrahydroxyethylethylenediamine§; ground opaque, fused silica‖ (over 50 per cent of the material passed through the No. 100 sieve and was retained by the No. 200 sieve); silicone R-23¶; and *o*-ethoxybenzoic acid, 2-methoxy-4-methylphenol, and *iso*eugenol (Eastman practical grade).

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†New Jersey Zinc Co.

‡Star Dental Co.

§Visco Products Co., Inc.

‖Thermal Syndicate, Ltd.

¶Silicone Division, Union Carbide and Carbon Co.

All other chemicals were reagent grade.

Methods.—

Consistency of mix: For some zinc oxide-EBA mixes the powder-liquid ratio of a mix of standard consistency was determined as outlined in American Dental Association Specification No. 9.[2] The consistency of the mix will depend on the technic of mixing as well as on the powder-liquid ratio. By changing such factors as the rate of spatulation and pressure it is possible to prepare mixes of standard consistency that show variation of 10 per cent in the amount of powder incorporated. Results obtained by different individuals may show variations as high as 10 per cent. However, it was found that such variations of the powder-liquid ratio will not alter appreciably the setting time of the mixes.

Setting time: The setting time is defined here as the number of minutes elapsed from the starting of the mix to the time when the point of a penetrating instrument such as the point of a Gilmore needle fails to make a perceptible indentation on the surface of the specimen. Setting times were determined either at room temperature or at 37° C.

Setting times of mixtures that harden in 10 minutes or longer are difficult to determine. Often, the mass of the mixture almost hardens within a few minutes, but on placing the needle on the surface of the material a small but still perceptible circle is produced even after many hours. The initial setting times reported in Tables III, V, VI, VII, and VIII refer to the time period after which only a slight indentation is visible after placing the needle on the material for 5 seconds. For a number of mixtures the time after which no perceptible circle is visible was determined as the final setting time.

It should be noted that the setting time depends largely on the experimental conditions used. Therefore different values were obtained when the tests were conducted at different temperatures and relative humidities.

When information was desired as to whether the mixes would harden at all and, if so, if they would set within a reasonable length of time, mixes were prepared containing the maximum amount of powder that the liquid would wet. The edge of a spatula was placed on the mix at various time intervals to determine the approximate setting time. Setting time was determined at room temperature (27±2° C.).

The setting times given in Tables III and V to X were determined using the amount of powder and liquid given in the tables. The powder-liquid ratios used were determined by previous experiments and give mixes of approximately standard consistency. Mixing was accomplished by placing weighed amounts of powder and 0.4 ml. of liquid on a glass slab. The powder was then divided into 4 portions. The first portion was completely incorporated with a stainless steel spatula before the next portion was brought in contact with the liquid. The mix was thoroughly spatulated until no unmixed powder remained. Since some mixes of standard consistency required a great deal of powder, the mixing time

to incorporate the powder varied from 2 to 4.5 min. Hence, the time at which the brass ring and mix was placed in an atmosphere of 100 per cent relative humidity at 37° C. was 3.5 ± 1.5 min. after starting the mix. With the exception of this modification, the setting time was determined with a 1 lb. standard Gilmore needle as described in A.D.A. Specification No. 9 for Dental Silicate Cements.[2]

Solubility and disintegration: For the solubility and disintegration test the powder and liquid were mixed in the same powder-liquid ratio by the procedure used for the setting time experiments. Since some experimental mixes adhere strongly to glass, pieces of Teflon were used between the specimens and the glass plates. After mixing the powder and liquid, 0.5 ml. of the mixture was transferred to a piece of Teflon, 0.03 mm. thick, and covered by a second piece of Teflon. These in turn were placed between two glass plates. The procedure followed thereafter is given in A.D.A. Specification No. 9.[2] Determinations were made in duplicate.

Compressive strength: Compressive strength was determined by the method outlined in A.D.A. Specification No. 9.[2] The specimens having the powder-liquid ratios given in Table V were made 3 ±2 min. after start of the mix by filling a hard rubber mold with the mixed cement. The ends of the mold were covered by pieces of Teflon, 0.03 mm. thick, and by flat glass plates held against the ends of the mold by a "C" clamp. The mold was placed in a bath at 37° C. and 100 per cent relative humidity for 1 hour. After removal of the glass plates and Teflon, the ends of the specimen were ground flat. The specimen was pressed out of the mold and immersed in water at 37° C. for 23 hr. Specimens were then crushed at the rate of 370 lb. per minute ±10 per cent.

Density: The density of the products was determined by using approximately 1 Gm. specimen in a 50 ml. pycnometer with water as displacement liquid.

Effect of zinc oxide-EBA products on polymerization: A few tests were conducted to evaluate zinc oxide-EBA mixtures as cement liners for acrylic resins. Mixes containing various proportions of eugenol-EBA and zinc oxide were placed in split molds, 6 mm. in diameter and 12 mm. in length. At time intervals ranging from 10 to 90 min. after placing the cement, room temperature curing resin was put on top of the cement by a "paint-in" technic.

RESULTS AND DISCUSSION

Reaction of Zinc Oxide With Various Chelating Agents.—Mixtures of zinc oxide and liquids that may be capable of forming chelated products were prepared. Results are given in Table I. *o*-Ethoxybenzoic acid reacts with reagent grade or Hyperfine zinc oxide to form a hard product in about 12 min. Zinc oxide-*o*-salicylaldehyde and -*o*-ethoxybenzoyl chloride mixtures harden fairly readily (1 to 2 hr.). A small percentage of Hyperfine zinc oxide incorporated in the reagent grade greatly speeds up the setting time of *o*-ethoxybenzoyl chloride mixes. Similar mixes with other chelating agents do not show this effect.

TABLE 1

REACTION OF ZINC OXIDE WITH CHELATING AGENTS AT ROOM TEMPERATURE

CHELATING AGENT	GRADE OF ZINC OXIDE	APPROXIMATE SETTING TIME (HR.)	PROPERTIES OF PRODUCT
eugenol	RG (a)	3.5	
eugenol	HF (b)	1.0	
*iso*eugenol	RG	(c)	
*iso*eugenol	HF	3	reddish, hard
guaiacol	RG	(c)	
guaiacol	HF	1	reddish, hard
2-methoxy-4-methylphenol	RG	16	hardens, slightly putty-like
2-methoxy-4-methylphenol	HF	2	fairly hard
o-ethoxybenzoic acid	RG	0.2	colorless, hard
o-ethoxybenzoic acid	HF	0.2	reddish, hard
o-ethoxybenzoyl chloride	RG	<1	yellow, fairly hard adhesive
o-ethoxybenzoyl chloride	HF	<1	reddish, sticky
o-ethoxybenzoyl chloride	95% RG 5% HF	0.03	yellow, hard, brittle
methyl salicylate	RG	<20	colorless cake, crumbly
methyl salicylate	HF	<20	reddish, soft
ethyl salicylate	RG	<18	colorless, coherent, crumbles
ethyl salicylate	HF	(c)	
*iso*amyl salicylate	RG	(c)	
*iso*amyl salicylate	HF	(c)	
o-salicylaldehyde	RG	1	yellowish-green, hard
o-salicylaldehyde	HF	2	brown, hard
o-methoxybenzaldehyde	RG	>70	grayish, putty-like
o-methoxybenzaldehyde	HF	(c)	
o-methoxyphenyl acetate	RG	(c)	
o-methoxyphenyl acetate	HF	(c)	red cake
o-nitroanisole	RG	(c)	
o-nitroanisole	HF	(c)	
veratrole	RG	>70	colorless, soft, coherent
veratrole	HF	(c)	
o-hydroxyacetophenone	RG	(c)	
o-hydroxyacetophenone	HF	<24	reddish, soft coherent cake
ethyl acetoacetate	RG	4	colorless, soft coherent cake
ethyl acetoacetate	HF	(c)	
ethyl benzoylacetate	RG	(c)	
ethyl benzoylacetate	HF	(c)	
acetylacetone	RG	0.02	colorless, hard
acetylacetone	HF	0.02	reddish, hard
citraconic anhydride	RG	1	chalky, coherent
citraconic anhydride	HF	(c)	soft coherent cake
lactic acid	RG	0.5	colorless, hard, disintegrates in water
lactic acid	HF	0.75	reddish, hard
pyruvic acid	RG	0.01	yellow, very brittle
pyruvic acid	HF	0.01	not cohesive, white fumes are given off during the reaction
ethoxyacetic acid	RG	<1	hard, brittle, adhesive
ethoxyacetic acid	HF	<1	reddish, hard, adhesive, disintegrates in water
ethylenediamine	RG	2	colorless, fairly hard, brittle
ethylenediamine	HF	4	reddish, fairly hard, brittle
tetrahydroxyethyl-ethylenediamine	RG	(c)	
tetrahydroxyethyl-ethylenediamine	HF	(c)	

(a)—Reagent grade.
(b)—Hyperfine.
(c)—Does not harden appreciably in 24 hours.

Some aliphatic chelating agents, especially those containing acidic groups (lactic acid, ethoxyacetic acid), and some compounds capable of enolization (acetylacetone) harden rapidly. The products are quite water soluble and usually disintegrate when placed in water. A number of compounds, such as esters of salicylic acid, ethyl acetoacetate, citraconic anhydride and ethylenediamine, form coherent products of relatively low compressive strength after a few hours. At room temperature under atmospheric conditions, mixes of eugenol, *iso*eugenol, guaiacol, and 2-methoxy-4-methylphenol harden more rapidly with Hyperfine than with reagent grade zinc oxide. It appears likely that the ammonia present in the Hyperfine material acts as an accelerator for mixes containing *o*-methoxyphenols. With the other chelating agents, differences in setting time and properties of the product vary little on substituting Hyperfine zinc oxide for the reagent grade.

From the small number of liquid chelating agents investigated, few conclusions can be drawn as to the effect of various substituent groups on the setting reaction. An acidic group (COOH or phenolic or enolic OH under certain conditions) appears to be necessary to obtain a hard coherent mass. Thus, *o*-ethoxybenzoic acid or guaiacol harden with zinc oxide whereas veratrole (*o*-dimethoxybenzene) does not react readily.

The mechanism of the reaction between zinc oxide and ethylenediamine is not known. The product formed in this reaction is undoubtedly of a complex nature.

Reaction of Various Oxides With o-Ethoxybenzoic Acid.—Since *o*-ethoxybenzoic acid appeared to be the most effective chelating agent, a study of the reaction of various oxides with this compound was undertaken. The results are summarized in Table II. Oxides of group IIA (MgO, CaO, BaO) of the periodic table react rapidly to form hard brittle materials. The oxides of group IIB (ZnO, CdO, HgO) form hard cohesive products as does lead oxide (litharge) in group IVB. The other oxides either do not react with *o*-ethoxybenzoic acid or form soft putty-like products.

Substituting magnesium oxide for zinc oxide in EBA mixes shortens the setting time and lowers compressive strength and increases considerably the solubility and disintegration (Table III). In air, the product adheres strongly to glass.

Partial replacement of zinc oxide with calcium oxide decreases the setting time (Table III). With some mixes, the setting time decreases to such an extent that not all of the powder and liquid can be thoroughly mixed before the mixture has set. Calcium oxide mixes result in an exothermic reaction. Where high percentages of calcium oxide are employed, the setting cement "boils" making the product very porous. Although no quantitative tests, with the exception of setting time, were conducted, it was apparent that addition of calcium oxide lowered the strength and resulted in high water solubility.

Reaction of Some Hydroxides, Halides and Zinc Salts with o-Ethoxybenzoic Acid.—

Results of the reaction of some metal hydroxides, chlorides and zinc salts with *o*-ethoxybenzoic acid are given in Table IV. Only the products formed

TABLE II

REACTION OF VARIOUS OXIDES WITH *o*-ETHOXYBENZOIC ACID AT ROOM TEMPERATURE

MATERIAL	APPROXIMATE SETTING TIME (MIN.)	PROPERTIES OF PRODUCT
Copper oxide	(b)	soft, putty-like
Silver oxide	(b)	no apparent reaction
Magnesium oxide	3	white, hard, brittle
Calcium oxide	<½ (a)	gray, very brittle
Barium monoxide	<½ (a)	pink-gray, hard, brittle
Zinc oxide (reagent)	11	hard, adhesive
Cadmium oxide	2-6	dark brown, hard
Mercuric oxide (yellow)	2-15	orange, hard, brittle
Mercuric oxide (red)	4	red, hard
Aluminum oxide	(b)	soft, putty-like
Titanium oxide	(b)	soft, putty-like
Stannic oxide	(b)	gray-white, cohesive, putty-like
Lead monoxide (litharge)	½	hard, yellow
Lead dioxide	(b)	black, soft, putty-like
Arsenic trioxide	(b)	soft, sticky, putty-like
Bismuth trioxide	(b)	greenish yellow, soft, putty-like
Molybdenum trioxide	(b)	soft, putty-like
Manganese dioxide	(b)	sticky, putty-like
Ferric oxide	(b)	soft, putty-like
Cobaltic oxide	(b)	black, putty-like
Nickelous oxide	(b)	green, cohesive, putty-like
Nickel dioxide	(b)	black, cohesive, putty-like

(a)—Highly exothermic reaction.
(b)—Does not harden appreciably within 24 hours.

TABLE III

REACTION OF VARIOUS OXIDES WITH *o*-ETHOXYBENZOIC ACID
TEMPERATURE: 37° C. RELATIVE HUMIDITY: 100%

OXIDE	STANDARD CONSISTENCY (GM./0.4 ML.)	INITIAL SETTING TIME (MIN.)	SOLUBILITY AND DISINTEGRATION (PER CENT)	COMPRESSIVE STRENGTH (PSI)
ZnO	2.30	13-24	7.97	7,200
MgO	0.65	3.5	48.5	low
75% MgO — 25% ZnO		3.5		low (brittle)
50% MgO — 50% ZnO	0.97	4.0	30.9	450
25% MgO — 75% ZnO		5.5		
CaO		0.5		
75% CaO — 25% ZnO		1.0		
50% CaO — 50% ZnO		1.0		
15% CaO — 85% ZnO		9.0		
10% CaO — 90% ZnO		40.0		
10% CaO — 50% MgO 40% ZnO		3.0		

with mercuric chloride have a hard cohesive mass which, however, is quite brittle.

Reaction of Zinc Oxide With o-Ethoxybenzoic Acid-Eugenol Mixtures.—Since preliminary studies showed that zinc oxide-EBA mixtures disintegrate slowly in water, a series of EBA-eugenol solutions was mixed with zinc oxide. Results of these studies are shown in Table V. Addition of even small amounts of EBA to eugenol increases markedly the amount of powder that can be incorporated to obtain a mixture of standard consistency (Fig. 1). Addition

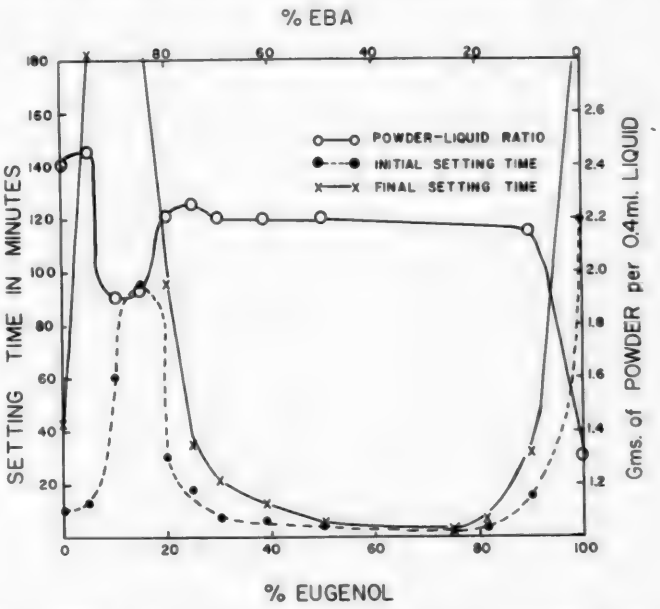

Fig. 1.—Setting time and powder-liquid ratio of zinc oxide-EBA mixes.

of EBA to eugenol decreases the setting time of the mixes (Fig. 1). A minimum initial setting time of 3 min. is obtained with 25 per cent EBA-75 per cent eugenol as compared to 120 min. for 100 per cent eugenol. The setting time remains short over the 20 per cent to 70 per cent EBA concentration range and increases rapidly on further increase in the EBA concentration. However, mixes containing 100 per cent EBA show a much lower setting time than those containing 15 per cent eugenol.

Addition of EBA increases the solubility and disintegration characteristics of the products from 0.10 per cent for zinc oxide-eugenol to 7.9 per cent for zinc oxide-EBA mixes (Fig. 2). In the solubility and disintegration tests, specimens made from zinc oxide-eugenol and EBA give highly viscous tarlike substances whereas zinc oxide-eugenol cements give white residues. Compressive strength increases on incorporating EBA. The maximum observed value of 10,600 psi occurs at a concentration of 75 per cent EBA (Fig. 2). Higher concentrations lower the compressive strength. The density increases from 2.68

TABLE IV

REACTIONS OF VARIOUS COMPOUNDS WITH *o*-ETHOXYBENZOIC ACID AT ROOM TEMPERATURE

MATERIAL	APPROXIMATE SETTING TIME (HR.)	PROPERTIES OF PRODUCTS
Hydroxides		
Lithium hydroxide	–	not very cohesive, fairly hard
Calcium hydroxide	(b)	not coherent (a)
Strontium hydroxide	(b)	soft, putty-like
Halides		
Sodium chloride	(b)	no apparent reaction
Magnesium chloride	(b)	no apparent reaction
Calcium chloride	75	mixture crumbles
Mercuric chloride	<24	hard, brittle
Lead chloride	(b)	yellow, putty-like
Zinc salts		
Zinc acetate	(b)	gray-white, cohesive, putty-like
Zinc oxalate	(b)	soft, cohesive, putty-like
Zinc sulfate	(b)	no apparent reaction

(a)—Exothermic reaction.
(b)—Does not harden appreciably within 24 hours.

TABLE V

PHYSICAL PROPERTIES OF *o*-ETHOXYBENZOIC ACID-EUGENOL-ZINC OXIDE MIXTURES
TEMPERATURE: 37° C. RELATIVE HUMIDITY: 100%

COMPOSITION (a)		POWDER USED FOR 0.4 ML. OF LIQUID (GM.)	SETTING TIME		SOLUBILITY AND DISINTEGRATION (PER CENT)	COMPRESSIVE STRENGTH (PSI)	DENSITY (GM./ML.)
SOLID	LIQUID		INITIAL (MIN.)	FINAL (MIN.)			
ZnO	100% EBA	2.30 (b)	13-24	88	7.97	7,200	
ZnO	95% EBA 5% Eugenol	2.35 (b)	13	182			
ZnO	90% EBA 10% Eugenol	1.90 (b)	60	1,040			
ZnO	85% EBA 15% Eugenol	1.95 (b)	95	860			
ZnO	80% EBA 20% Eugenol	2.20 (b)	30	95			
ZnO	75% EBA 25% Eugenol	2.25 (b)	18	35	3.21	10,600	3.31
ZnO	70% EBA 30% Eugenol	2.20 (b)	7.0	21			
ZnO	62.5% EBA 37.5% Eugenol	2.20 (b)	6.5	13			
ZnO	50% EBA 50% Eugenol	2.20 (b)	4.0	5.5	2.31	4,400	
ZnO	25% EBA 75% Eugenol	1.70	3.0	3		low, (crumbles)	
ZnO	17.5% EBA 82.5% Eugenol	1.75	3.5	8			
ZnO	10% EBA 90% Eugenol	2.10 (b)	16	33			
ZnO	100% Eugenol	1.30 (b)	120	540			
ZnO	100% Eugenol (commercial product)	1.40 (b)	–	8	0.1	3,000	2.68
99.5% ZnO 0.5% $Zn(Ac)_2$	EBA	2.30	10	43	–	–	–
ZnO	Guaiacol	1.6	60	–	0.076	–	–

(a)—Percentage by weight for solids and by volume for liquids.
(b)—Powder-liquid ratio that yields mix of standard consistency.

for zinc oxide-eugenol to 3.31 Gm./ml. for mixes containing 75 per cent of EBA. No quantitative tests were conducted on the adhesive properties of the products. However, the behavior of the materials during the tests indicates that addition of EBA increases the adhesive properties.

The effect of the zinc oxide particle size on the powder-liquid ratio and setting time of mixes containing a 75 per cent-EBA in 25 per cent-eugenol solution is given in Table VI. It will be noted that the powder-liquid ratio used for a standard consistency mix decreases with decreasing particle size. It appears that the setting time is more dependent on the method of manufacture of the zinc oxide than on the dimensions of the zinc oxide particles. Thus, at 37° C. and 100 per cent relative humidity, the EBA-Hyperfine mix hardens slowly whereas zinc oxide No. 513 has the shortest final setting time. The greater reactivity of the No. 513 zinc oxide is probably due to the presence of 2.5 to 3 per cent of carbonate in the material.

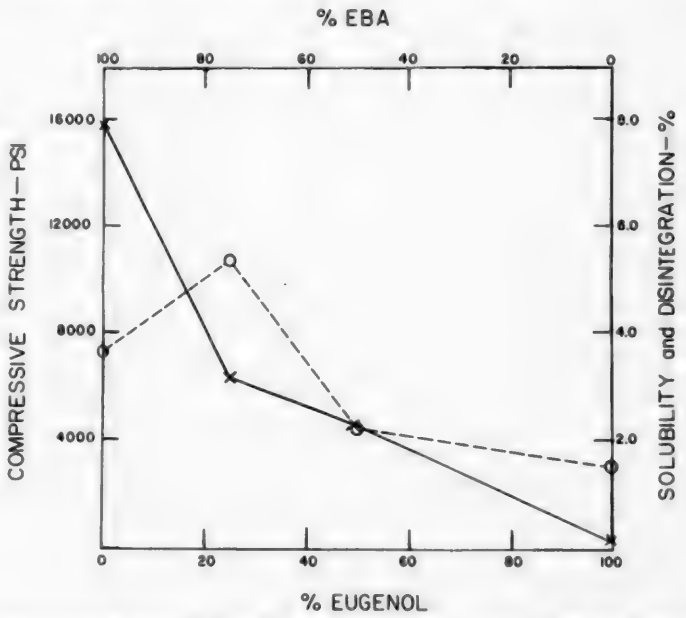

Fig. 2.—Compressive strength and solubility and disintegration of zinc oxide-eugenol-EBA mixtures. x————x solubility and disintegration. O- - - - -O compressive strength.

The effect of addition of fillers to zinc oxide-EBA-eugenol mixes on the setting time and on the physical properties of the products was also investigated. Results of these studies are shown in Tables VII and VIII and Fig. 3.

Addition of fused silica increases the total amount of powder which can be incorporated to yield a mix of approximately standard consistency (Table VII). The quantity of zinc oxide in the mix is not altered appreciably by the addition of the fused silica. Setting time of the mixes is shortened and can be further decreased by the addition of 0.6 per cent of zinc acetate accelerator.

TABLE VI

SETTING TIMES OF EBA-EUGENOL SOLUTIONS WITH ZINC OXIDES OF VARYING PARTICLE SIZE

TEMPERATURE : 37° C.
RELATIVE HUMIDITY: 100%
LIQUID : 75% EBA
25% EUGENOL

GRADE OF ZINC OXIDE	AVERAGE DIAMETER OF PARTICLE (a) (MICRONS)	SURFACE AREA (a) M.²/GM.	STANDARD CONSISTENCY (GM./0.4 ML.)	SETTING TIME		REMARKS
				INITIAL (MIN.)	FINAL (MIN.)	
Reagent	–	–	2.30	24	88	–
XX-78	0.3	4	2.15	26	72	–
Kadox-72	0.2	6.5	2.05	34	77	–
Kadox-15	0.1	10	1.80	36	81	–
U. S. P.	–	–	1.60	34	70	–
Micronized U. S. P.	–	–	2.70	25	81	–
No. 513	0.04	31	0.25	26	54	very brittle
Hyperfine	0.02	–	0.50	55	113	–

(a)—Values given by the manufacturer.

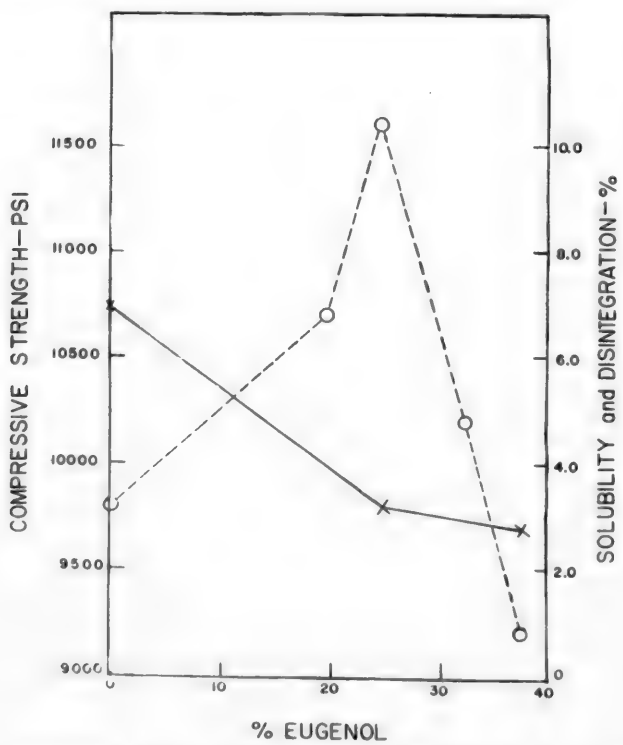

Fig. 3.—Compressive strength and solubility and disintegration of zinc oxide-fused silica-EBA-eugenol mixtures. Powder: 69.2% ZnO-30.8% fused silica. Liquid: EBA-eugenol. x————x solubility and disintegration. O- - - - -O compressive strength.

Products of slightly higher compressive strength are obtained. A mixture containing 69.2 per cent of zinc oxide, 30.8 per cent of fused silica, and 75 per cent of EBA-25 per cent of eugenol has a compressive strength of 11,600 psi (Fig.

3). Solubility and disintegration of the products are little affected by incorporation of fused silica in the mixes. Addition of 5 per cent of silicone R-23 to the 75 per cent-EBA in 25 per cent-eugenol liquid does not change the setting time but decreases the solubility and disintegration of the products from 3.2 per cent to 2.5 per cent.

TABLE VII

PHYSICAL PROPERTIES OF *o*-ETHOXYBENZOIC ACID-EUGENOL-ZINC OXIDE-FUSED SILICA MIXES
TEMPERATURE : 37° C.
RELATIVE HUMIDITY: 100%

COMPOSITION (a)				POWDER USED FOR 0.4 ML.	SETTING TIME		SOLUBILITY AND DISINTEGRATION	COMPRESSIVE STRENGTH	DENSITY
SOLID		LIQUID							
ZnO (%)	SILICA (%)	EBA (%)	EUGENOL (%)	LIQUID (GM.)	INITIAL (MIN.)	FINAL (MIN.)	(PER CENT)	(PSI)	GM./ML.
69.2	30.8	100	—	3.20	10.0	19	7.0	9,200	
69.2	30.8	80.0	20.0	3.00	6-11	51		10,700	
69.2	30.8	75.0	25.0	3.25	8.0	32	3.2	11,600	2.87
69.2	30.8	70.0	30.0	3.00	5-10	21		10,200	
69.2	30.8	62.5	37.5	3.00	4.5-7	7	2.81	9,200	
68.8	30.6	75.0	25.0	2.60	4.0			10,300	
+0.6% $Zn(Ac)_2$									
67.2	29.8	75.0	25.0	2.70	3.0			8,000	
+3% $Zn(Ac)_2$									
69.2	30.8	75.0	25.0	3.25		32	2.5		
		+5.0% Silicone R-23							
100	—	72.5	22.5					6,900	
		+5% Silicone R-23							

(a)—Percentage by weight for solids and by volume for liquids.

Addition of dark rosin as filler shortens the setting time slightly and lowers the compressive strength considerably (Table VIII). For this reason, no solubility and disintegration tests were conducted.

On the addition of tricalcium phosphate as a filler (Table VIII), much less powder can be incorporated into mixes of approximately standard consistency. The crushing strength of the product is lowered.

Calcium oxide or magnesium oxide when incorporated with zinc oxide, eugenol, and EBA give a fast setting mix (Table VIII). The product has a smooth surface, but has low crushing strength.

In an effort to decrease the solubility of the hardened product, the readily available 2,4-dimethoxybenzoic acid (2,4-DMBA) was incorporated into some mixes containing quartz (Table VIII). 2,4-DMBA is only slightly soluble in eugenol or EBA. However, addition of a small amount of DMBA to 50 per cent eugenol-50 per cent EBA increases the solubility of the products. A mixture of 25 per cent eugenol-75 per cent EBA saturated with 2,4-DMBA hardens after addition of zinc oxide in 38 min. The compressive strength of the cement is lowered to 6,500 psi and the cement appears less adhesive when compared to the products containing no 2,4-DMBA. Incorporation of small amounts of 2,4-DMBA does not lower the solubility.

Addition of solutions of 2,4-DMBA in methanol or ethanol to eugenol-EBA and zinc oxide produces mixtures that do not harden within 18 hours, even when liberal amounts of zinc acetate are incorporated to act as accelerator.

TABLE VIII

PHYSICAL PROPERTIES OF *o*-ETHOXYBENZOIC ACID-EUGENOL-ZINC OXIDE MIXES CONTAINING VARIOUS ADDITIVES

COMPOSITION (a)		POWDER USED FOR 0.4 ML. OF LIQUID (GM.)	INITIAL SETTING TIME (MIN.)	SOLUBILITY AND DISINTEGRATION (PER CENT)	COMPRESSIVE STRENGTH (PSI)
SOLID	LIQUID				
69.2% ZnO 30.8% $Ca_3(PO_4)_2$	75.0% EBA 25.0% Eugenol	1.08			6,000
81.8% ZnO 18.2% Rosin	75.0% EBA 25.0% Eugenol	2.75	6.5		4,400
50.0% ZnO 50.0% MgO	50.0% EBA 50.0% Eugenol		3		low
75.0% ZnO 25.0% MgO	25.0% EBA 75.0% Eugenol		5.5		
40.0% ZnO 50.0% MgO 10.0% CaO	50.0% EBA 50.0% Eugenol		4		
66.0% ZnO 33.0% Silica 1.0% DMCA	72.7% EBA 27.3% Eugenol	2.20	12.5		7,800
68.8% ZnO 30.6% Silica 0.6% $Zn(Ac)_2$	50.0% EBA 50.0% Eugenol +2,4-DMBA	2.60		3.7	
69.2% ZnO 30.8% Silica	75.0% EBA 25.0% Eugenol sat. with 2,4-DMBA	2.25	38		6,500

(a)—Percentage by weight for solids and by volume for liquids.

2,3-Dimethoxycinnamic acid (2,3-DMCA) was also incorporated into eugenol-EBA-zinc oxide mixes (Table VIII). 2,3-DMCA is soluble in either eugenol or EBA and can be mixed with either or both before addition of zinc oxide. Addition of 1 per cent 2,3-DMCA appears to increase the setting time and lower the crushing strength.

It may be possible to lower the solubility of the products of the zinc oxide-eugenol-EBA reaction by incorporation into the mixture tailor-made ethoxybenzoic acid derivatives. Such compounds should be liquids containing hydrophobic side chains at the unsubstituted positions of the ring. Synthesis of such compounds in this laboratory and investigation of their effect on setting are contemplated.

Chelating Agents as Accelerators of Zinc Oxide-Eugenol Mixtures.—Crowell[3] has shown that addition of small quantities of acetic acid to eugenol greatly speeds up the setting reaction of zinc oxide-eugenol mixtures. Therefore, the effect of a 5 per cent addition of a number of chelating agents on the setting times of the mixes and compressive strength of the products was investigated. Results of the setting time measurements at 37° C. and 100 per cent relative humidity are given in Table IX. Addition of acetic acid or a chelating agent increases the amount of powder that can be incorporated in the mix. All compounds, with the exception of ethylenediamine, accelerate the reaction. Compounds containing carboxylic acid groups give the shortest setting times. The compressive strength of the products is low.

TABLE IX

PROPERTIES OF ZINC OXIDE-EUGENOL MIXTURES CONTAINING ACETIC ACID OR CHELATING AGENTS
TEMPERATURE : 37° C.
RELATIVE HUMIDITY: 100%

COMPOSITION OF LIQUID		POWDER USED PER 0.4 ML. LIQUID (GM.)	FINAL SETTING TIME (MIN.)	COMPRESSIVE STRENGTH (PSI)
EUGENOL (PER CENT)	ADDITIVE (PER CENT)			
100		1.30	540	3,000
99.5	0.5 acetic acid	2.25	12	1,800
95	5 acetic acid	2.25	6	3,500
95	5 ethoxyacetic acid	2.35	12	2,400
95	5 lactic acid	2.25	24	—
95	5 acetylacetone	2.35	73	—
95	5 ethyl acetoacetate	2.25	90	—
95	5 ethylenediamine	2.25	>200	—

The effect of addition of acetic acid or chelating agents to EBA is less pronounced (Table X). Addition of these compounds lowers the powder-liquid ratio as well as the setting time.

TABLE X

SETTING TIMES OF ZINC OXIDE-EBA MIXTURES CONTAINING ACETIC ACID OR A CHELATING AGENT
TEMPERATURE : 37° C.
RELATIVE HUMIDITY: 100%

COMPOSITION OF LIQUID: 95% EBA
5% ADDITIVE

ADDITIVE	POWDER USED PER 0.4 ML. LIQUID (GM.)	FINAL SETTING TIME (MIN.)
EBA	2.30	88
Acetic acid	1.20	35
Ethoxyacetic acid	1.90	55
Acetylacetone	2.00	57
Ethylenediamine	2.10	>60

Effect of Zinc Oxide-EBA-Eugenol Mixtures on the Polymerization of Acrylic Resins.—

Commercial self-curing acrylic monomer-polymer slurries placed over cements containing more than 5 per cent eugenol do not harden. With cements containing 100 per cent EBA or 95 per cent EBA-5 per cent eugenol, the acrylic slurry hardens rapidly in all cases. However, monomer from the acrylic softens the cement.

Acrylic resins also harden when placed over salicylaldehyde zinc oxide mixtures. The cement is not readily attacked by the monomer. When this cement is placed in cavities of extracted teeth, the teeth are stained yellow, probably due to the presence of the aldehyde group.

SUMMARY

Mixes of zinc oxide with many compounds capable of forming chelates result in coherent products. Mixes containing *o*-ethoxybenzoic acid, salicylaldehyde, acetylacetone, *o*-ethoxyacetic acid or lactic acid form hard products within 1 hour at room temperature. Some of these products disintegrate in water.

EBA and oxides of group IIB and those tested in group IIA of the periodic table as well as lead oxide harden rapidly.

Solutions of EBA and eugenol, when mixed with zinc oxide, have a short setting time. The products show a marked increase in compressive strength. Water solubility and density of the product increase.

Addition of quartz powder further improves the physical properties of the hardened material.

The products may be of interest as dental impression pastes or temporary fillings.

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THE SEX DIFFERENCE IN TOOTH CALCIFICATION

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DURING the years of growth and development, girls are advanced over boys in a great many respects. They are earlier, on the average, in appearance of ossification centers; they are ahead in epiphysial union and the appearance of secondary sexual characteristics. While the direction of sexual dimorphism in the deciduous dentition may be somewhat questionable,[1] the fact that girls tend to be earlier in eruption of the permanent teeth is not. For white children of European ancestry, the average sex difference in the time of eruption is 0.45 year (or nearly 5 per cent) with a maximum of 0.93 year (9 per cent) for the mandibular canine tooth.[2]

Whether comparable or equivalent sex differences exist in tooth calcification is open to question. The most commonly used standards fail to provide an answer. The norms of Logan and Kronfeld,[3, 4] based on histologic examinations of jaw sections of 30 children, make no distinction between the sexes; Broadbent's roentgenographic standards similarly lump male and female data.[5] While the roentgenographic investigations of Gleiser and Hunt on the mandibular first molar[6] and Demisch and Wartmann on the mandibular third molar[7] both suggest advancement of girls over boys, generalization cannot be made from their studies. It is entirely possible that dental advancement of girls over boys is largely limited to eruption, so that the permanent teeth of girls emerge into the mouth with a lesser degree of calcification. Since, in the course of the Fels Longitudinal investigations, we have amassed serial roentgenograms on a sizable sample of children, exceeding in number the sum of previous studies combined, it seemed pertinent to investigate in detail the question of sex differences in those stages of tooth development not easily accessible to visual examination. The matter of a sex difference and the magnitude of this difference were the problems of primary interest in this investigation.

METHODS AND MATERIALS

The present study is primarily based upon serial oblique-jaw x-rays of 255 white children from southwestern Ohio, regularly enrolled in the Fels Longitudinal studies,[8] and born between the years 1928 and 1953. The oblique-jaw series, supplemented by lateral skull plates, was begun in 1939.

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For each child, and for the mandibular molar and premolar teeth, the age at which each of 5 stages of calcification and eruption was first noted was recorded as shown in Fig. 1.

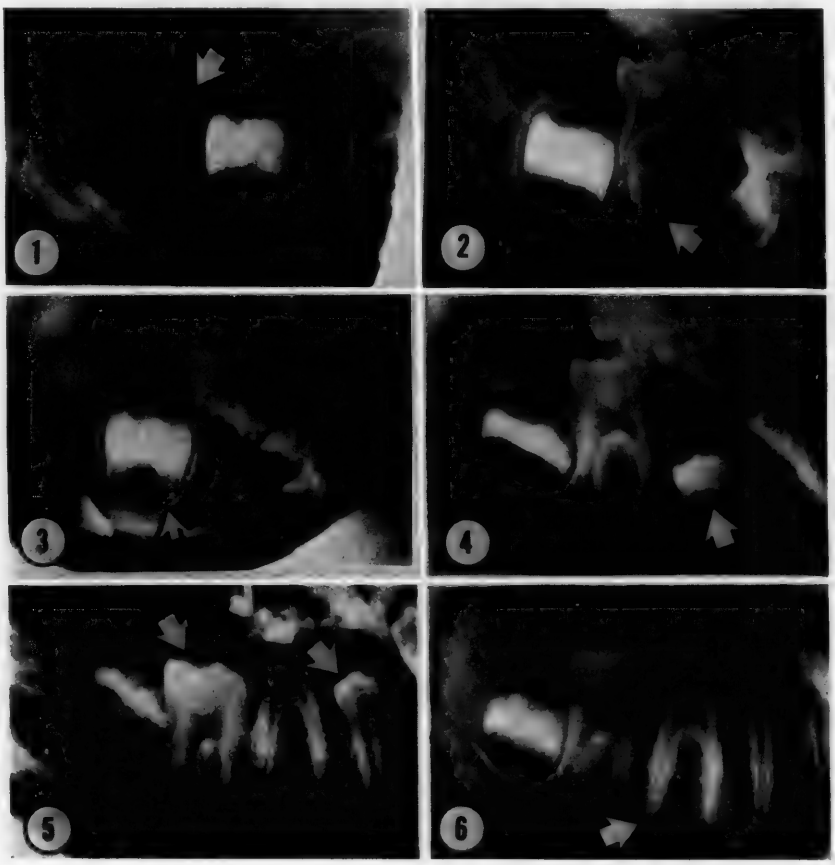

Fig. 1.—Reproductions of oblique-jaw radiographs showing stages of calcification and eruption utilized in the present study. *1*, full-follicle stage of M_2. *2*, full-follicle stage of P_2. *3*, beginning root formation of M_2. *4*, beginning root formation of P_2. *5*, alveolar eruption, P_2 and M_2. *6*, attainment of occlusal level P_2 and M_1. Apical closure of M_1 (shown by arrow).

Stage I.—Stage of the full-follicle, immediately preceeding the first evidence of cusp calcification, and therefore systematically 6 months earlier than the latter in each case.

Stage II.—Crown completion and beginning root formation, especially as characterized by the "floor" in molar teeth (Fig. 1).

Stage III.—Alveolar eruption, i.e., elevation of the crown above the alveolar margin. This stage is reasonably comparable to alveolar eruption as commonly determined in skeletalized material.[9]

Stage IV.—Attainment of the occlusal level.[10]

Stage V.—Apical closure.

These 5 stages were selected as constituting a reasonable number of definite and fairly well-spaced events in the developmental course of the teeth considered, and representing both calcification (Stages I, II, and V) and eruption (Stages III and IV). While a far larger number of stages could be identified,[6] such detail seemed superfluous for the purposes of the present study. Interpolation was avoided throughout.

In connection with the statistical analyses, it was first necessary to preclude existence of secular trends.* However, upon sorting the data according to year of birth, there was no evidence that the later-born Fels children were either earlier or later in tooth calcification and eruption. Again, since many developmental phenomena are positively skewed, with respect to age, the arithmetic means were compared to the medians for all stages of all teeth considered; the difference between the two measures of central tendency was negligible throughout. Finally, Snedecor's F test[11] was employed to determine whether one sex was more variable than the other, with essentially negative results. Accordingly, the usual *t* test for significance of the differences between means was then employed.

It will be noted that sex comparisons of Stage I (full-follicle) of M_1, and the Stages III, IV, and V of M_3 are omitted from the tables. Since the majority of boys and girls had achieved Stage I of M_1 by one month, the time of the first x-ray in this series, it was not possible to compute norms for this stage of M_1. Further, the number of boys and girls who had attained the later stages of M_3 formation and eruption was, at the time of writing (November, 1957), too small to permit comparisons. It is hoped that supplemental data on M_3 may be presented at a later date.

FINDINGS

As shown in Table I, where age-means and average sex differences are given for the mandibular molar and premolar teeth, girls tend to be advanced over boys by an average of 0.32 year, and by amounts up to 0.92 year (10.1 months). Of the total of 21 stages of calcification and eruption compared, sex differences were significant by the *t* test[11] in 10. In 17 of the sex comparisons, the girls were advanced over the boys, whereas in 4 there was no difference or the boys had an earlier mean age of eruption. Thus, the obtained proportion of 17:4, compared to the chance proportion of 10.5:10.5 yielded a Chi-squared value of 23.5, which is significant at better than the 1 per cent level of confidence.[11] There is little question, therefore, that girls tend to be advanced in the formation, as well as in eruption, of these posterior teeth.

Comparing the 2 eruption stages (alveolar eruption and attainment of the occlusal level) to the calcification stages (follicle and beginning root) the sex difference of 0.52 year for the former is greater than the average sex difference of 0.16 year for the latter. However, when the sex differences were expressed as a percentage of the mean age for each stage, the differences were not significant. Throughout, they averaged 3 per cent. Absolute sex differences

*The term "secular trends" refers to the widespread tendency for children to be larger and earlier-maturing in successive decades or generations.

widened as the children grew older, but remained constant percentagewise, as shown in Fig. 2. Thus, later stages of tooth development necessarily evidenced greater sex differences than earlier stages for the same teeth.

Again, as shown in Fig. 2, the equation $y = 0.97\ x$ describes the sex difference for 21 stages of tooth calcification and eruption as determined in the present study. This is the same order of magnitude as the equation $y = 0.95\ x$ as computed by Gleiser and Hunt[6] from Hurme's normative data on the eruption of all permanent teeth,[2] with the exception of M_3. Notably, while these different measures of dental maturation agree closely in the extent of sexual dimorphism, measures of bony maturation yield quite different results. The osseous maturation of the knee, using standards 9 through 26 in Pyle and Hoerr,[12] evidenced a greater sex difference, y being 0.81 x. For the wrist,

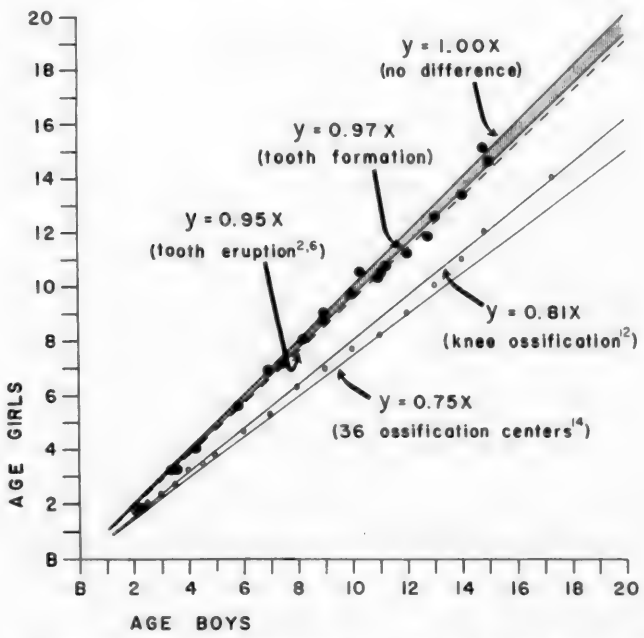

Fig. 2.—Comparison of sex differences in tooth formation, tooth eruption, and two measures of osseous maturation. Sexual dimorphism in dental maturation is quite small ($y = 0.97\ x$ and $0.95\ x$), whereas sex differences in the maturation of the knee and the time of appearance of 36 bony centers are considerably larger ($y = 0.81\ x$ and $y = 0.75$). Data from Hurme,[2] Gleiser and Hunt,[6] Hoerr and Pyle,[12] Sontag and Pyle,[14] and Table I of this paper.

using 48 maturity indicators covering the ages 1 to 16 years, and taken from Greulich and Pyle,[13] the comparable value of K was 0.88. And finally, 38 ossification centers in the knee, hand, shoulder, upper arm, hip, and foot gave evidence of greater difference between the sexes, y being equal to 0.74 x (using norms published by Sontag and Pyle[14] and Sontag and Reynolds[15]).

It follows, then, that girls are earlier than boys on the average, in tooth calcification, alveolar eruption, and attainment of the occlusal level. However, sex differences in dental development, while marked, are of a lesser order

TABLE I
SEX DIFFERENCES IN TOOTH CALCIFICATION AND ERUPTION

TOOTH AND STAGE		BOYS NO.	BOYS MEAN AGE	GIRLS NO.	GIRLS MEAN AGE	SEX DIFFERENCE (YEARS*)
P_1	I	98	2.2	66	1.8	0.34
P_1	II	97	7.4	75	7.2	0.23
P_1	III	46	10.1	47	9.7	0.39
P_1	IV	34	10.9	34	10.3	0.59
P_1	V	24	13.0	16	12.5	0.48
P_2	I	100	3.3	79	3.3	0.00
P_2	II	90	8.2	76	8.0	0.19
P_2	III	33	11.1	31	10.3	0.76
P_2	IV	27	12.2	23	11.3	0.92
P_2	V	18	14.1	14	13.4	0.75
M_1	II	105	4.3	70	4.0	0.25
M_1	III	54	5.8	39	5.7	0.11
M_1	IV	46	6.9	24	6.9	0.02
M_1	V	48	10.3	39	10.6	-0.21
M_2	I	113	3.5	83	3.2	0.31
M_2	II	89	8.9	88	8.7	0.21
M_2	III	40	11.2	39	10.7	0.59
M_2	IV	26	12.7	17	11.8	0.88
M_2	V	22	15.0	15	14.6	0.42
M_3	I	69	9.0	66	9.0	-0.01
M_3	II	34	14.6	19	15.2	-0.55

Sex difference significant at $p = 0.05$ or better by t test and Table D in Guilford.[11]
*Computed from means before rounding off.

of magnitude than the degree of sexual dimorphism in osseous maturation. There is at least three times as much of a sex difference in bony maturation as there is in dental development over the same time period.

DISCUSSION

It is evident from these findings that girls tend to be advanced over boys, not just in eruption of the teeth into the mouth (as is commonly known) but, also, in tooth calcification and eruption from the alveolus. Thus, the present data confirm the trend suggested by the work of Gleiser and Hunt[6] and of Demisch and Wartmann[7] and point to the need for quantitative sex-specific norms for tooth calcification.

Expressed in absolute units, namely years and tenths of a year, there is a suggestion that the sex difference is greater for alveolar and gingival eruption than for calcification. However, when the differences are related to age, either as a percentage of the mean age for a particular stage, or to the logarithm of age, this indication no longer holds. The sex difference is simply greater for the later stages of tooth development; girls are approximately 3 per cent ahead of the boys throughout. This value approximates that for the sex difference in gingival eruption but is considerably smaller than the sex difference in carpal ossification (8 per cent), ossification of the knee (19 per cent), or appearance of centers (25 per cent).

Whenever a sex difference is considered, the possibility of hormonal mediation suggests itself. Many of the maturational differences between boys and girls can be attributed either to the early secretion of gonad-stimulating

hormones in girls, or to unique end-organ responses to particular sex hormones. Moreover, there is some evidence of a relationship between hormonal activity and tooth eruption. Methyl testosterone administered to the castrate rhesus monkey dramatically accelerates canine eruption.[16] Sexually advanced children are earlier in M_2 eruption, as well as being larger and fatter.[17] In the present study there are fair to good correlations (0.4 to 0.6) between M_2 eruption and age at menarche.*

However, if sex differences in tooth formation and eruption were primarily dependent upon sex hormone activity, we should expect little sexual dimorphism before the tenth year. Actually, the sex difference exists well before that time, being, as we have shown, the same relative magnitude as the later differences. Moreover, precocious puberty (*Pubertas praecox*) and endocrine disorder, markedly advancing sexual development, do not appear to be associated with advanced dental development.[18, 19, 20] One reservation may be stated, however; it is possible that the negative findings reported to date are due to the limitations of the Logan-Kronfeld norms customarily employed.[3]

While it would be desirable to know why the sex difference in tooth calcification and eruption is small (3 to 5 per cent) as contrasted with far larger sex differences in skeletal maturation (10 to 25 per cent), there is a prior problem to answer, namely, why such differences exist at all. To say that tooth size, for example, is in some way influenced by sex is merely restating the observations. To suggest that the teeth are less responsive to the factors that bring about sexual differentiation, casts no additional light upon the fundamental problem.

SUMMARY

1. Sex differences in tooth calcification were investigated in a total of 255 native-born white children from southwestern Ohio, using serial oblique-jaw x-rays taken over a period of 18 years.

2. In general, girls tended to be advanced over boys, in the mean ages at which each of 5 distinct stages of calcification and eruption were attained.

3. The smallest sex differences were observed early in life, with the absolute divergence between the sexes increasing steadily. On the average, girls were 3 per cent ahead of boys.

4. While the sex differences observed here were of the same order of magnitude as those known to exist for tooth eruption (5 per cent), they were considerably smaller than the sex differences in osseous development (10 to 25 per cent).

5. Since girls were advanced over boys well before the tenth year of life, the sex difference in tooth formation could not be attributed primarily to differences in the timing of sex hormone secretion.

6. These findings indicated the need for sex-specific, quantitative norms for tooth development.

*The correlation between aveolar eruption (Stage III in the present study) of M_2 and age at menarche was 0.60 for 28 girls. The correlation between attainment of the occlusal level (Stage IV) and menarche was 0.44 for 15 girls. In both cases, the obtained correlation was significant at $p = 0.05$ or better.

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